EXHIBIT O

DOSE-RESPONSE RELATIONSHIPS IN NITROSAMINE CARCINOGENESIS

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Dedicated to my wife

Margaret

for making it

possible.

Summary

The current state of knowledge relating to nitrosamine carcinogenesis is reviewed. An animal study is described which is designed to determine the dose-response pattern for two nitrosamines (diethyl and dimethyl nitrosamine) over a wide dose range using a large number of animals and dose-groups.

The results of this study are given with details of tumours, their incidences, sites and pattern in time. These results are analysed to show that although there is an apparent threshold of response at low doses this is due to the failure of the animals to survive to a time when any incidence of tumours would be expected. It is also concluded that incidence only decreases with decreasing dose as a result of the interaction of normal mortality patterns with the incidence pattern of tumours.

Finally the implications of some of the results are discussed and the analysis extrapolated to provide an estimate of risk for man from known exposure to low concentrations of nitrosamines.

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INDEX

INDEX TO FIGURES

INDEX TO FIGURES - continued

INDEX TO FIGURES - continued

INDEX TO TABLES

INDEX TO TABLES - continued

CHAPTER ONE

INTRODUCTION

Nitrosamine Carcinogenesis

N-Nitroso compounds are to be found in food [Gough et al., 1977], in certain alcoholic beverages [Spiegelhalder et al., 1979; Goff and Fine, 1979], in cigarette smoke [Brunneman et al., 1978; Webb and Gough, 1980], and in various industrial processes [Fan et al., 1977; Stephany et al., 1978; Webb and Gough, 1980; Fine, 1980]. Additionally N-nitroso compounds can be formed within the body by the interaction of nitrite and secondary or tertiary amines or amides [Lijinsky, 1981]. The evidence for the carcinogenicity of many nitroso-compounds in a wide range of animal species [Schmahl, 1981; Bogovski and Bogovski, 1981] leads to concern about the consequences of the known human exposure to these compounds. The N-nitroso compounds can be conveniently divided into two broad categories, the N-nitrosamines which require metabolic activation before demonstrating carcinogenic properties and the N-nitrosamides which do not require such activation.

Presence of N-nitroso compounds in food is frequently correlated with the use of sodium nitrite as a preservative. The N-nitroso compounds arise from the interaction of the nitrite and secondary or tertiary amines present in the food [Challis, 1981]. The production of some of these N-nitroso compounds, particularly N-nitroso pyrrolidine in bacon is significantly enhanced by cooking [Gough et al., 1978], although approximately 90% of that produced volatilises during the cooking process [Gough et al., 1976]. Diets that contain no preformed N-nitroso compounds may give rise to nitrosated products following ingestion [Walters et al., 1979]. These are again formed from nitrite and secondary or

tertiary amines, although there is evidence that dietary nitrite may be supplemented by the conversion of nitrate to nitrite both by plant enzymes and bacteria (White, 1975).

In addition to dietary sources of secondary or tertiary amines or amides there is much concern about the fate of many drugs which have such structures and are known to be nitrosated '*in vivo*' in a number of animal species (Lijinsky, 1981).

Although some N-nitroso compounds are not carcinogenic their production in the body cannot be regarded as without risk as trans-nitrosation reactions are known to occur in which the N-nitroso group is transferred to a different secondary amine, possibly giving rise to a carcinogenic product (Dennis *et al.*, 1979).

The potential of the human exposure to these chemicals to produce cancer is unknown and in view of effects in animals is worthy of investigation. Before going on to explore alternative ways of investigating this potential it is important to consider the nature of cancer and its possible origins.

The Nature of Cancer

Cancer is a collective name for a variety of proliferative lesions possessing certain common properties:

A. GENETIC DAMAGE - The change present in the cells is heritable and generally irreversible and the lesion does not regress in the absence of the causative stimulus.

B. INVASION - Surrounding tissue is invaded or infiltrated by cancer cells.

C. METASTASIS - cells from the original tumour may be transported to distant sites where they settle and give rise to secondary colonies of cancer cells.

The main difference between a chemically-induced cancer and any other toxic lesion is its ability to progress in the absence of further stimulus. The persistence of the change together with its heritability is generally considered to indicate that the ultimate carcinogenic mechanism involves changes in the genetic material of affected cells.

The changes in DNA commonly associated with treatment with chemical carcinogens are alkylations of nucleic acids. Particular sites on particular nucleic acids show a high frequency of alkylation [Faustman and Goodman, 1981]. Although in certain cases there is a tendency for a correlation of alkylation at a specific site on DNA with carcinogen-potency at that site, this is not universally the case. The site of alkylation showing the best correlation with target specificity and potency is the alkylation of the O_6 position of guanine [Loveless, 1969; Pegg, 1977]. These workers demonstrated that a significant factor in determining site of carcinogen action was the rate of repair of the O_6-alkylated guanine. However, as in many areas of research, it is one thing to demonstrate a correlation between two events, but is far more difficult to demonstrate a causative relationship between them. Thus, while O_6 alkylation of guanine may play a part in chemical carcinogenesis it may not be the step or even the pathway that brings about cell transformation.

This is in accord with a view expressed by Cairns [1981] who, in reviewing the role of mutation in carcinogenesis

concluded that the genetic changes involved in cancer production are major structural alterations, possibly involving changes in the expression of parts of the genome. While it is clear that the mutations which may result from local damage, such as alkylation, may contribute to the major structural alterations referred to, they are not sufficiently significant, in themselves, to bring about the changes in cell behaviour that are typical of cancer.

It is conceivable that some parts of the genetic material are more susceptible to damage than others and there is some evidence that alkylation of chromosomes is not uniform [Faustman and Goodman, 1981]. One theory of control of gene expression postulates the existence of control elements,' sequences of DNA which, according to their state permit or prevent subsequent genes being coded. If one of these control elements were damaged by carcinogens then the effect is likely to be much greater than damage at other sites. Such an event might lead to expression of genes not normally typical of the cell type. This idea would tend to fit in with observations made by Klein [1981] in a review of work with transforming viruses. This review suggests that cell transformation may result from the activation of a single 'oncogene' which forms part of the DNA of all cells.

Thus, in some way, the ability of all carcinogens to bind to DNA or to generate radicals which do so can bring about major changes in the genetic material which cause cell transformation. The precise mechanism awaits a greater understanding of the normal processes of control of gene expression.

If the only step in carcinogenesis was DNA damage then

cancer would follow exposure to carcinogens very rapidly. Even at the highest non-toxic doses of the most potent carcinogens known, some weeks must elapse between first treatment and appearance of the first tumour. In known human cases of chemical carcinogenesis, cancer does not usually appear until many years after first exposure. Thus, whatever change has taken place in the genetic material lies apparently dormant until triggered by other processes or events. These two stages have been defined as Initiation and Promotion. The carcinogen is essential for initiation and generally can act as a promoter, however, promotion may be achieved by a much wider range of stimuli. Recent work on autoxidative changes resulting from free-radical generation in the cells suggests a possible mechanism whereby the two stage process may be achieved. If it is assumed, as appears to be the case, that a cell with altered DNA continues, in the absence of other stimuli, to function as a relatively normal member of the population from which it is derived, then the essential step to a cancer cell might be achieved by a gradual alteration of the rest of the cell to provide the environment in which the altered DNA exerts its transforming properties. Such an alteration of non-nuclear cell components is known to result from carcinogen treatment, with alkylation of a whole range of cellular macromolecules. One specific change that has been identified, following carcinogen treatment is abnormal glycoprotein synthesis (Warren et al., 1978). As glycoproteins form an essential part of cell membranes and particularly of cell-surface tissue-specific sites, they have a significant role in preserving cell and tissue identity.

It is conceivable that this change in the cell may be prevented or, at least repaired in cells with undamaged nuclei. In cells with altered DNA however these changes may accumulate until a suitable environment is created for the altered DNA to act to stimulate cell growth and division just as if the surrounding tissue did not exist. Some support for this idea comes from work on cultured cells. It is widely accepted that many cell cultures are more susceptible to cell transformation than the parent tissue and that such transformation occurs almost immediately after treatment with the carcinogen. It is possible that these cells in culture are not subject to the normal range of tissue-specific factors, and as such are closer to the state which may be achieved by promotion.

There is evidence that treatment with high doses of carcinogens kills some cells or groups of cells within an organ, while others which are then resistant to subsequent treatment with the carcinogen, survive. It may be that these 'selected' cells are genetically altered and, if so, present ideal targets for the epi-genetic effects of the carcinogen. There is much evidence from the pattern of development of some epithelial tumours, from hyperplasia through benign to malignancy, for a gradual process of carcinogenesis rather than a number of dramatic steps or stages. Such a process could be the gradual loss of cell identity with increasing dominance of abnormal glycoproteins which eventually would allow a genetically altered cell to transform.

Chemical Carcinogenesis

Agents known to be capable of causing cancer are:

a) Ionising and ultra-violet radiation.

b) Reactive chemicals including free-radicals.

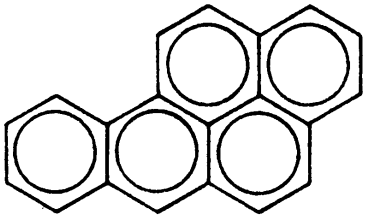
c) Viruses.

While much effort has been devoted to discovering viral origins for human cancer, this has produced little evidence for viruses as a major cause. Suspicion has therefore fallen upon chemicals, as a potential cause of many apparently 'spontaneous' human cancers.

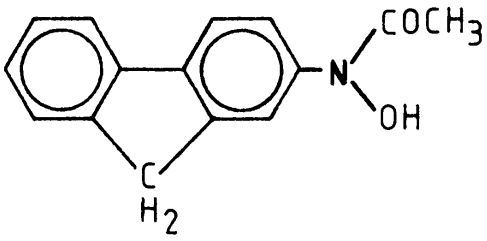
The variety of chemicals known to be capable, at high doses, of inducing tumours in animals does nothing to allay the suspicions. The mechanisms by which chemicals with widely diverse structures bring about very similar changes in the target tissues is worthy of discussion, for it is based on an understanding of these mechanisms that any conclusions concerning human exposure can be drawn.

A number of examples of carcinogens are shown in figure 1.1.

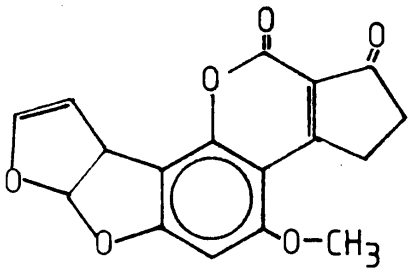
The first factor that all these different structures have in common is the requirement for metabolism, generally occurring on the endoplasmic reticulum. The postulated proximal carcinogens in each case are shown in figure 1.2. Each of these is a highly active molecule which will tend to bind to cellular macromolecules or to generate radicals that do so. Thus a common feature of chemical carcinogens is the ability to give rise to covalently bound adducts of all cellular macromolecules including DNA. Certain molecules within the cell possess a great affinity for these active



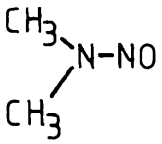
BENZO(a)PYRENE



2-ACETYLAMINOFLUORENE



AFLATOXIN B_1

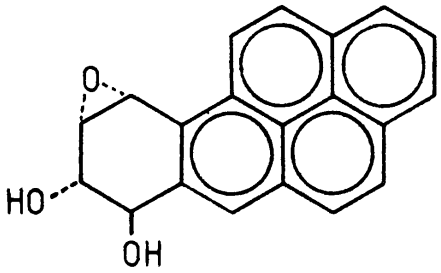


DIMETHYLNITROSAMINE

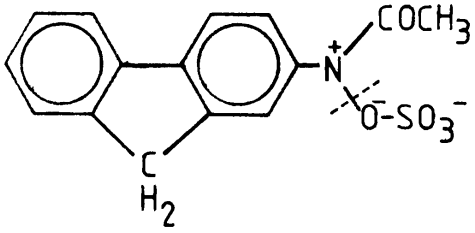
FIGURE 1.1 THE STRUCTURES OF SOME CHEMICAL CARCINOGENS

Figure 1.2 This figure shows the structure of the proximate carcinogens which are produced within the body from the carcinogens shown in figure 1.1.

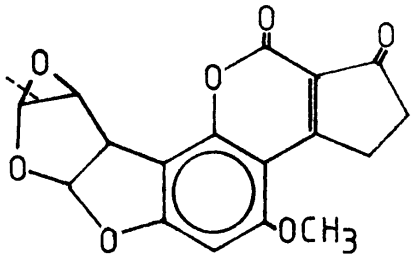
CARCINOGEN	PROXIMATE CARCINOGEN
Benz[a]pyrene	[a] Benzo[a]pyrene 7,8-diol-9,10-oxide
2-Acetylaminofluorene	[b] 2-Acetylaminofluorene-N-sulphate
Aflatoxin B_1	[c] Aflatoxin B_1 2,3-oxide
Dimethylnitrosamine	[d] Methyldiazonium ion.



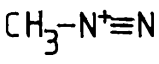
(a)



(b)



(c)



(d)

FIGURE 1.2 STRUCTURES OF PROXIMATE CARCINOGENS

molecules, and may, under normal circumstances act to reduce the formation of covalent adducts with other molecules. Examples of such 'scavengers' are glutathione, retinol and vitamin E. Other factors such as DNA repair may also act to alter the effect of a dose of carcinogen. While it is known that man is susceptible to certain chemical carcinogens, this evidence arises from circumstances where exposure is high, such as occupational or drug treatment. There is still a need to discover what risk, if any, is posed by those chemicals, known to be capable of causing cancer in animals, and known to be present in the human environment.

Methods of Determination of Risk to Man from Carcinogens

An approach which attempts to study this problem directly is that of epidemiology. By studying cancer incidence in different populations it is possible firstly to decide whether any population faces a different risk of cancer from the rest. If so, some cause for this difference can be sought. While this approach is useful in suggesting associations between particular habits or diets and cancer incidence there is little chance of ever deriving a certain cause and effect relationship. Thus it is known that populations in South America and parts of China who have high nitrate and nitrosamine intakes respectively also demonstrate unusually high incidences respectively of gastric and oesophageal cancer. While it is not possible to draw firm conclusions from this data due to the difficulty of isolating single factors, it must act to guide other experimental approaches.

The main experimental method, at present, for determining

whether or not a chemical is a carcinogen is by administration over the life-span to laboratory animals (generally rats and/or mice), at doses ranging up to the highest dose compatible with normal growth and survival of treated animals. Interpretation of a positive result in such studies presents many problems, based in a general uncertainty as to the suitability of any animal species as a model for man, and much debate over the correct method of deriving quantitative conclusions for man from such data. However, short of experimenting on humans, the animal studies are the best method available, and by understanding the process involved, it is possible to minimise the uncertainty.

Various short-term investigations designed to predict carcinogenic potential have been proposed and some have achieved usefulness, however such tests struggle to duplicate the results of animal studies and thus do not offer a better way of predicting human risk. Most such studies are based on detecting the mutagenicity of a chemical, a property which appears to be closely linked to its carcinogenic potential.

In the future, studies of mutagenicity in different human populations may tell us more about risk of cancer but this data is not yet available and will not be for some time.

Nitrosamines - Carcinogens for Animals

The story of nitrosamine toxicity begins as do many others with an observation of the effects of human exposure. Two laboratory workers were found to have developed cirrhosis of the liver following approximately 10 months' exposure to dimethylnitrosamine (DMN) which was being used as a solvent.

This observation prompted investigations of acute and short-term toxicity by Barnes and Magee (1954). The animal species used in the acute investigations were rats, mice, rabbits, guinea-pigs and dogs. In all these species the LD_{50} appeared to be between 10 and 30 mg/kg. In all species the liver was the target organ, showing various degrees of damage accompanied by peritoneal exudate, haemorrhagic in the case of rats, dogs and guinea-pigs. Animals surviving a single dose for up to 10 days showed similar signs of hepatic damage. A repeated dose study was confined to rats given doses of 50, 100 and 200 ppm in the diet for 110 days. The groups receiving 100 and 200 ppm died before the end of the study.

The histological picture in all acute cases showed centrilobular necrosis with haemorrhage into the necrotic areas. Animals surviving the acute dose for more than 10 days showed evidence of regeneration of liver tissue from the cells of the periportal areas. In the repeated dose study centrilobular necrosis persisted with some evidence of abnormal nuclear forms in the surviving periportal cells.

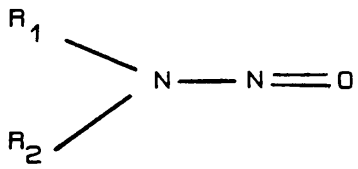
The same authors in 1956 went on to study the longer term effects of DMN in rats and demonstrated its carcinogenic properties. A group of 10 male and 10 female rats were fed a diet containing 50 ppm DMN. A control group of 5 males and 5 females received basal diet. The first treated animal died after 27 wk of treatment and by wk 42 only one of this group remained alive. As in the acute studies, the only organ to be affected was the liver, where 19 of the 20 treated animals developed primary tumours. The main lesions seen in

the livers were classified as hepatomas which appeared to originate from hepatocytes and included a range of types from advanced hyperplastic nodules to metastasising anaplastic tumours. A second type of lesion was encountered, described at autopsy as small translucent cysts. These were considered to be of biliary origin and to be a separate lesion from the hepatomas, being described as cystadenomata.

Following these observations a large number of nitrosamines have been studied in a wide range of species. In ma instances, they are carcinogens each with specific target organs, in a given species, although the target organ for a single nitrosamine can vary from one species to another as illustrated in data prepared by Schmähl [1981] for diethylnitrosamine.

Nitrosamines - Structure, Metabolism and Mechanism of Action

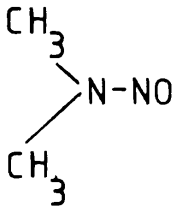
All nitrosamines possess the following structure:



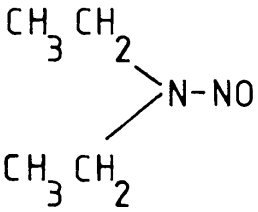
where R_1 and R_2 are aryl or alkyl groups and may be identical, different, or part of a ring system. Some examples are given in figure 1.3.

The lower members of the dialkyl series, together with some cyclic nitrosamines are steam-volatile, and can be quantitatively recovered from complex mixtures and body

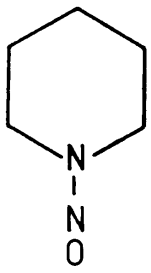
FIGURE 1.3 SOME EXAMPLES OF N-NITROSAMINES



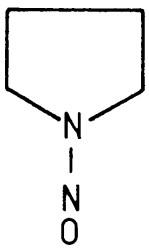
DIMETHYLNITROSAMINE



DIETHYLNITROSAMINE



N-NITROSOPIPERIDINE



N-NITROSOPYRROLIDINE

tissues and fluids for analysis. As quantitative extraction of the 'non-volatile' nitrosamines has not yet been achieved successfully most research is restricted to the volatiles. This is not too great a disadvantage as the most potent carcinogens are the two lowest members of the dialkyl series, diethylnitrosamine (DEN) and dimethylnitrosamine (DMN).

Studies of structure-activity relationships for nitrosamines have suggested correlations between carcinogenicity and water-hexane partition coefficients (Wishnok et al., 1978) and chain-length or number of carbon atoms (Wishnok and Archer, 1976). These two factors may illustrate the effects of transport and metabolism respectively on the carcinogenic potency of a given nitrosamine.

The most frequently studied nitrosamine metabolism is that of DMN. Despite many years of work by a large number of independent groups the metabolism of this nitrosamine is poorly understood. It is known that administration of DMN leads to alkylation of cell macromolecules, including DNA (Magee and Barnes, 1967), and that an administered dose is rapidly broken down to CO_2 (Heath, 1962). As for many chemicals, it was suspected that the metabolism of nitrosamines involved an initial oxidation by enzymes of the microsomal mixed function oxidase system dependent upon cytochrome P_{450}. However, experiments based on this supposition failed to give entirely consistent and reproducible results (Lake et al., 1974, 1976). Recent work by Lake et al. (1978) has pointed to a route for metabolism involving N-oxidation by an amine oxidase which is independent of cytochrome P_{450}. It is possible that this pathway runs in

parallel with a P_{450}-dependent oxidation. Beyond this point it is postulated that degradation proceeds non-enzymically to yield carbonium ions which are the active alkylating species, along with formaldehyde, methanol and nitrogen. Although it has been a temptation for many researchers in this field to assume that the metabolism of DMN must proceed via an alkylating mechanism, it is clear that this does not need to be the major pathway. This view is supported by the results of Cottrell et al. (1977) who demonstrated in studies using ^{15}N-labelled DMN that production of nitrogen was only 5% of that expected if DMN were metabolised as predicted. More recent data (Milstein and Guttenplan, 1979; Kroeger-Koepke et al., 1981) have concluded that nitrogen production is almost quantitatively what is expected. This anomaly awaits resolution.

There is evidence from studies with inhibitors of amine oxidase activity that inhibition of this enzyme during DMN treatment affords protection from the carcinogenic properties of this nitrosamine (Hadjiolov, 1971; Schmähl et al., 1976; Weisburger et al., 1974).

Thus, in summary, the main route of toxicity and carcinogenicity of nitrosamines is concluded to be the alkylation of cell components. It is therefore the rate at which the alkylating species is generated in each tissue which determines the target organ and the potency of each nitrosamine.

Nitrosamines - Human Exposure and Hazard?

There is no direct evidence for the carcinogenicity of any nitrosamine for man. There is, on the other hand, no

reason to believe that man is any less susceptible to the carcinogenic effects of nitrosamine than any other species studied. Human exposure to nitrosamines is composed of two elements. Firstly, the ingestion or absorbtion of preformed nitrosamines present in various foodstuffs, tobacco smoke and industrial chemicals. Secondly nitrosamines are formed in the body from secondary amines and nitrite.

Highly sensitive and specific analytical techniques have demonstrated nitrosamines in many foods preserved with or containing nitrite (e.g. bacon, cheese, ham) (Spiegelhalder et al., 1980) in alcoholic beverages (Spiegelhalder et al., 1980) in tobacco smoke (Brunneman, 1980) industrial cutting oils (Webb and Gough, 1980) and a variety of other sources. The general exposure to preformed nitrosamines from dietary sources has been calculated to be 1.1 µg/day for DMN and 0.1-15 µg/day for N-nitroso pyrrolidine (NPYR) (Spiegelhalder 1980); 1 µg/week for DMN and 3 µg/week for NPYR and N-nitroso-piperidine (NPIP) (Gough et al., 1978); 0.38 µg/day DMN (Stephany and Schuller, 1980). While there is some difference between these figures a range of 0.1-1 µg/day for DMN and other alkylnitrosamines and 0.1-15 µg/day for NPYR and NPIP includes all the figures. There is a contribution to nitro-samine intake from cigarette smoking and exposure to side--stream cigarette smoke (Brunneman et al.,1980) and from certain occupations such as those involving leather tanning, rocket-fuel production and tyre chemicals. Excluding these avoidable exposures an upper limit of 10 µg/day expressed as DMN should cover the average human exposure to preformed nitrosamines.

The other major source of nitrosamines in the general population is the in vivo production from dietary amines, and drugs combining with nitrite. Nitrosation of secondary and tertiary amines will proceed without enzyme assistance on incubation with sodium nitrite at low pH (Andrews et al., 1980). As these conditions may exist in the stomach a number of animal studies have been carried out to discover the effect of simultaneous dietary administration of several amine drugs and sodium nitrite (Lijinsky, 1981). Not only was nitrosamine formation demonstrated but these studies also continued for long enough to show that the combination of amine and nitrite was, in some cases, carcinogenic.

While these studies demonstrate that carcinogenic nitrosamines can be formed 'in vivo', the concentration of nitrite is artificially high. Thiocyanate is known to be a powerful catalyst for nitrosation (Boyland and Walker, 1974) and is present in normal human gastric juice (Ruddell et al., 1977). It is therefore conceivable that nitrosamines could be formed in the normal human stomach after ingestion of food or drugs containing tertiary or secondary amines. This formation would be considerably enhanced by the simultaneous ingestion of nitrite or nitrate. This formation is likely to be enhanced in individuals having a low basal level of acid secretion since this appears to be correlated with higher gastric nitrite concentration and colonisation of the stomach by bacteria capable of catalysing nitrosation (Ruddell et al., 1976). Analysis of human urine samples has demonstrated the presence of low levels of nitrosamines (Kakizoe, 1979) while analysis of faecal samples detected no nitrosamines (Lyang-ja

Lee, 1981). The detection of nitrosamines in urine may lend support to the view that the urinary bladder represents a second potential site for 'in vivo' nitrosamine formation, particularly when infected (Radomski et al., 1978).

It is likely that the rate-limiting factor in human 'in vivo' nitrosamine formation is the nitrite concentration. Due to the ability of oral bacteria to catalyse nitrite formation from nitrate, it is likely that human populations most at risk from nitrosamines formed in vivo are those exposed to high dietary nitrite and/or nitrate concentrations. Evidence for such a connection has been generated in a study of a population in Colombia exposed to an unusually high nitrate intake, and known to have an exceptionally high incidence of gastric cancer. Hawksworth et al. (1974) detected only low levels of gastric nitrite in this population and concluded that high urinary nitrate levels were the source of nitrosamines which subsequently caused the gastric cancer. The precise course of events in this population has not been established. However, it is generally accepted that nitrosamine formation is a major contributary factor to the excess cancer risk (Tannenbaum, 1981).

Thus, human exposure to nitrosamines is impossible to estimate accurately and is likely to vary considerably from one individual to another. It is unlikely that exposure would exceed a total of 100 µg/day from all sources. In any attempt to calculate risk for man from nitrosamine exposure the relative risks of exposure to 10 µg/day and 100 µg/day will be compared.

Nitrosamines as Model Chemical Carcinogens

For many people cancer is a very emotive word, and the desire to see cancer conquered is very strong in our society. When well-known scientists indicate that up to 80% of cancer may have an environmental cause, and in the same breath identify a range of chemicals known to cause cancer, it is not surprising that many people believe that all cancer is caused by chemicals.

This is clearly not true, however, the belief has generated considerable pressure to reduce exposure to carcinogens. Before this can be achieved, we must know which chemicals are carcinogens and which are the most potent. In this way attention can be directed at the major hazards. The evidence cited in the preceding pages for the carcinogenicity of many nitrosamines in a range of animals species, and their possible involvement in at least some human cancers identifies these chemicals as deserving of investigation. A number of questions exist relating to the sensitivity of man to these compounds compared with animal species studied, and the precise level of exposure. One general question is also worthy of consideration; this relates to the quantitative difference between all animal studies and likely human exposure and the possibility that at very low levels of exposure there is no effect. This possible 'Threshold' of effect is dependent upon one or a combination of the following:

(i) Failure of transport mechanisms below a certain concentration.

(ii) Shift in metabolism to 100% detoxication at low doses.

(iii) Immediate and total repair of cell damage.

In order to shed some light on the significance of nitrosamines as human carcinogens, some complete understanding of the effects in animals must be achieved. One part of this understanding is the clarification of the quantitative relationship beyween rate of exposure to the carcinogen and the incidence rate of the induced tumours over the widest possible dose-range. The following is an account of such a study carried out in rats, investigating the carcinogenicity of a wide range of doses of dimethyl and diethylnitrosamine.

CHAPTER TWO

STUDY DESIGN

Concept of Study

Faced with a decision to carry out an animal study and finite space and funds, the effects of low doses of nitrosamines can be studied in one of two ways.

The first approach is to expose a small number of groups of animals to the dose-levels of interest. The number of groups is limited by the available space, as the groups themselves must be large to detect the low expected incidence of tumours.

The second approach is to measure the response of animals to a wide range of doses of nitrosamine. In this way the relationship between dose and response may be established, and the likely response at the low doses deduced from this relationship.

Each approach has its merits and disadvantages. The first studies the problem directly, but the accurate detection of an increased incidence of tumours requires more animals than could be employed. The second approach is based, necessarily, on the assumption that a relationship established between dose and response at high doses will hold good at the dose-levels of interest. It was decided to use a large number of dose-groups spanning a wide range of dose, as this approach was considered to stand more chance of providing useable data, than the large-group low-dose study, which might provide no information at all.

Selection of Species

As the ultimate aim of this study is an understanding of human exposure it is necessary to ensure that the species

used makes this extrapolation as simple as possible. Two basic factors therefore affect species choice:

a) Availability - the animals must be available in sufficient numbers from an adequately defined population.

b) Experience - it is highly desirable that the value of the species selected, as a model for human carcinogenesis, is known. Similarly, the background pathology in the species chosen should be well-established.

These limit the choice of species to three; rat, mouse and hamster. Of these, the hamster has been used by relatively few workers and is not known to be of any better predictive value than rat or mouse. Mice are available in a much greater variety of strains than rats. However, the variability, between strains, in background incidence of a number of tumours makes the rat the species of choice for these studies, by default.

The next stage of the process was to define the particular strain of rat that would be used. To aid this decision a number of further factors were considered:

a) Inbred Strain - in an experiment setting out to quantify an already established response the aim is to minimise variability in the population studied.

b) Experience - the strain must have been used in chronic studies, spanning at least 2 years, to provide the necessary background knowledge of spontaneous tumour incidences and age-related pathology.

c) Availability - sufficient animals must be available for the study.

d) Low tumour incidence - it would be preferable that the chosen strain had a low spontaneous incidence of the tumour types likely to be induced by treatment. Similarly it would be preferable if the strain were not unusually susceptible to any specific type of tumour.

The only strain that satisfied all the above conditions, at the time of designing this study, was the Colworth Inbred Wistar from Unilever Ltd., Colworth, Sharnbrook, Beds.

Selection of Nitrosamines

It was necessary to choose representative examples of this group of chemicals as it was impossible to study the whole range. Diethyl (DEN) and dimethyl (DMN) nitrosamines were selected as being the most potent carcinogens in the group while being, structurally, the simplest members of the dialkyl series. For similar reasons they have also been the subject of considerable study by a number of workers (Druckrey, 1967; Terracini et al., 1967; Magee and Barnes, 1956).

Group Sizes, Dose-levels and Schedules

Based on data from previous studies (Druckrey, 1967; Terracini, Magee and Barnes, 1967) it was decided to use a dose-range between 50 and 25,600 ppb in the diet. The lowest dose is considered to be of a similar magnitude to the highest doses encountered by man; whereas the highest dose is known to produce tumours in rats, but with minimal

toxic effect.

The major limitation on the number of groups was the available space. Accommodation was available for approximately 4,000 rats. Thus with a group size set at 50 of each sex, being the smallest number considered capable of giving a useful measure of response, 40 groups could be accommodated. It was decided that the study should contain a large control group to generate as much contemporary background data as possible. The design ultimately decided on was of a control group containing 240 males and 240 females and 15 dose-levels of each nitrosamine, each containing 60 males and 60 females, adding up to 4080 rats. The lowest dose shown to produce tumours by previous workers (Druckrey, 1967) was approximately 1000 ppb in diet. As the establishment of a dose-response relationship depends on the availability of a measurable response it was decided to bias the dose-range so that ten of the groups received doses in excess of 1000 ppb. This was achieved as below:

Original dose-groups	Interpolated groups
25600 ppb	
12800 ppb	
	9600 ppb
	8000 ppb
6400 ppb	
	4800 ppb
	4000 ppb
3200 ppb	
	2400 ppb
1600 ppb	
800 ppb	
400 ppb	
200 ppb	
100 ppb	
~~50 ppb~~	

Preliminary investigations of the stability of DEN and DMN in diet at the highest of the above concentrations showed losses exceeding 20% of the added amount within 24 hours. Stability in water solution was found to be considerably better and therefore the drinking-water was selected as the route of administration. It was known from many previous studies that rats drink a greater weight of water each day than they consume diet. From historic data it was determined that multiplication of the concentrations intended for dietary administration by 0.66 would give the concentration in the drinking water that would be likely to provide a similar mg/day dose, The resultant dose-levels were thus:

33, 66, 132, 264, 528, 1056, 1584, 2112, 2640, 3168, 4224, 5280, 6336, 8448, 16896 ppb in the drinking-water.

The numbers of animals required for this study were too large to consider starting the whole study at one time. It was therefore decided to divide the study, to ensure effective management at all stages. Each dose-group, including the controls was divided into 10 sub-groups such that each sub-group contained 24 controls of each sex and 6 of each sex for each dose of each nitrosamine.*
Each sub-group commenced treatment on a different occasion as shown below:

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*In sub-group 6 the female 16896 DEN group was omitted and the female 2112 DEN group consisted of 12 animals.

Sub-group	Day of birth†	Day of first treatment	Age (days at first treatment
1	1-11	48	37-48
2	15-25	60	35-45
3	30-40	75	35-45
4	44-54	89	35-45
5	56-68	104	36-48
6	86-96	133	37-47
7	99-110	147	37-48
8	113-123	161	38-48
9	127-137	175	38-48
10	156-166	203	37-47

The first two sub-groups were taken for interim kill at 12 months and 18 months from the start of treatment, respectively.* All the remaining animals were allowed to continue until they qualified for autopsy, either by death, sickness, or positive palpation. Treatment continued until autopsy in all cases. Animals were kept in their original cages without regrouping for the whole study. The following observations were made for each animal during each week of the study:

Body weight

Presence of palpable abdominal lump

General condition.

For each cage of animals the amount of water/solution consumed was measured weekly.

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† Day 1 is defined as the earliest day of birth of sub-group 1.

* Results from the groups terminated at 12 and 18 months are excluded from further analysis.

At autopsy the following data was recorded:

Date

Dose-group

Animal number

Sex

Reason for autopsy

Details of all macroscopic abnormalities.

On microscopic examination of all the tissues taken at autopsy details were recorded of all histopathological changes seen.

Based on all the available information the most likely cause of death was determined for each animal.

CHAPTER THREE

EXPERIMENTAL METHODS AND MANAGEMENT

ENVIRONMENT CONTROL

Due to the extremely low concentrations of nitrosamine under investigation in this study, the diet, drinking-water, general accommodation and maintenance procedures for the animals were given much attention, in order to identify and minimise any additional variables.

Animal Accommodation

All rats were housed in cages constructed from stainless steel and polyethylene. These cages were held in racks, each containing fifteen cages. Excreta were collected on high wet strength kraft paper, held on trays beneath the cages.

The animals were kept in four rooms of an animal unit given over specifically to this study. The division of the study between the rooms is shown in figure 3.1. Air was provided to the animal rooms at a rate sufficient to give 15 air changes per hour in each room. The cages were arranged in the room in such a way that the air was always being drawn away from the control animals, thus minimising possible contamination. This air was filtered to remove particles down to 1 mμ in size. Although the air supply unit contained no provision to reduce air temperature below ambient, the temperature of the animal rooms was maintained at 21 $\pm$ 2°C., apart from a few isolated occasions when it reached 27°C. Humidity was maintained between 50 and 80%.

Lighting of the rooms was on a 12-hour light, 12-hour dark cycle. The light part of the cycle coincided with the working day.

Figure 3.1 Distrubition of study in animal rooms and coding system employed.

ROOM 1 Colour code BLUE DIETHYLNITROSAMINE				ROOM 2 Colour Code GREEN DIETHYLNITROSAMINE				ROOM 3 Colour code RED DIMETHYLNITROSAMINE				ROOM 4 Colour code YELLOW DIMETHYLNITROSAMINE			
Conc's (ppb)	Dose code	No. of rats ♂	♀	Conc's (ppb)	Dose code	No. of rats ♂	♀	Conc's (ppb)	Dose code	No. of rats ♂	♀	Conc's (ppb)	Dose code	No. of rats ♂	♀
33	1	60	60	2100	1	30	30	33	1	60	60	2100	1	30	30
66	2	60	60	2600	2	60	60	66	2	60	60	2600	2	60	60
130	3	60	60	3200	3	60	60	130	3	60	60	3200	3	60	60
260	4	60	60	4200	4	60	60	260	4	60	60	4200	4	60	60
530	5	60	60	5300	5	60	60	530	5	60	60	5300	5	60	60
1060	6	60	60	6300	6	60	60	1060	6	60	60	6300	6	60	60
1580	7	60	60	8400	7	60	60	1580	7	60	60	8400	7	60	60
2100	8	30	30	16900	8	60	60	2100	8	30	30	16900	8	60	60
Control	0	60	60	Control	0	60	60	Control	0	60	60	Control	0	60	60
Total		510	510	Total		510	510	Total		510	510	Total		510	510
		1020				1020				1020				1020	

Animals

Rats were transported weekly in cardboard transport boxes with soft-wood shavings as bedding, from Colworth to BIBRA. Food and water were not provided in the transport boxes. Each box contained animals of a single sex weaned on a single day, and was marked with this date.

On arrival at BIBRA the sex of each animal was checked and they were caged in groups of 10-15, each cage containing animals weaned on the same day. These cages were provided with pelletted diet (page 36) and distilled water.

When sufficient animals had been collected for the next phase of the experiment to commence and acclimatised at BIBRA for at least 7 days, the following procedure was used to assign animals at random to treatment group: on each occasion of randomisation the animals were to be allocated to 31 treatment groups, one (control) required 24 rats whereas all others required 6 rats. Lists of random sequences of numbers from 1 to 31 were generated for the purpose by Richard Peto (Oxford). The lists were employed as below:

A cage of rats was emptied into a clean plastic bucket. A rat was taken from the bucket and allocated to the treatment groups designated by the first number on the list. The tail of the rat was colour-coded as the first animal in that cage and the weaning date was recorded. The next animal was taken from the bucket and assigned to the treatment-group designated by the second number on the list etc. A different colour was used to mark the tail of the 1st, 2nd, 3rd, 4th, 5th and 6th rats in each cage. Once all

the cages contained the required number of rats the animals were permanently identified using an ear-punch code (Fig 3.2).

Animal Maintenance Procedures

The paper used for collecting excreta was changed daily. All cages were washed on a 3-weekly rota. When animals were transferred from one cage to another, this was carried out so that only one cage was open at any one time. The cages for each treatment group were labelled with waterproof marker to ensure that there was no exchange of cages between groups. The coding system used was a combination of colours and numbers identified in Figure 3.1. The use of the coding is best illustrated by examples:

4200 ppb DEN	A figure 4 in black on a green background
530 ppb DMN	A figure 5 in black on a red background.

Diet

Three commercially available diets suitable for the maintenance of rats were analysed for content of steam-volatile nitrosamines and aflatoxin.† The results of these analyses are shown in Table 3.1.

At this stage it was discovered that future production of the Unilever diet was uncertain and this was withdrawn

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†The invaluable assistance of the laboratory of the Government Chemist, and the Tropical Products Institute, in performing these analyses is gratefully acknowledged.

FIGURE 3.2 RODENT EAR MARKING CODE

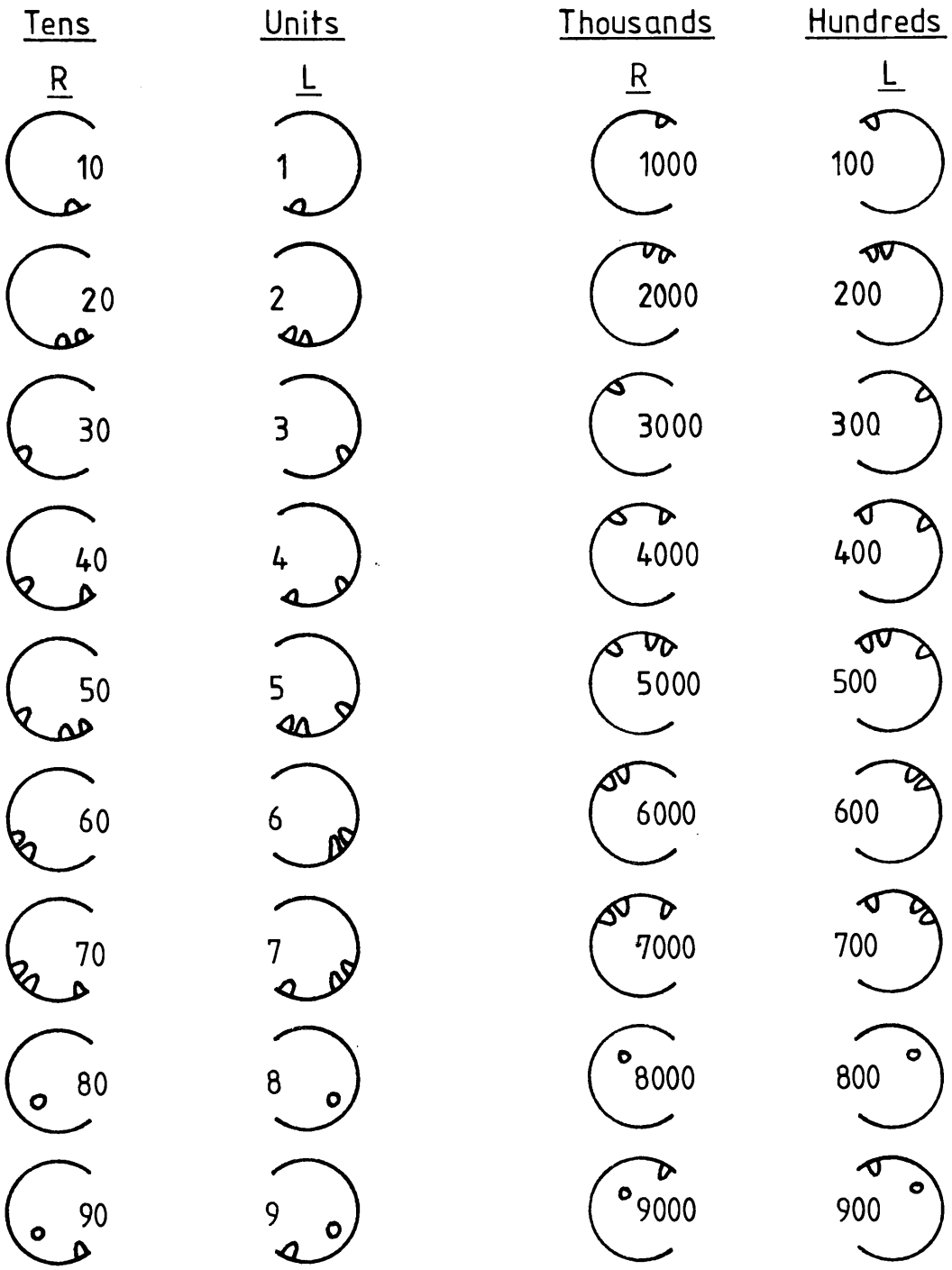


Table 3.1 Analysis of commercial rodent diet for nitrosamines

Diet Source	DMN (ppb)	Other nitrosamines (ppb)
Spillers	5-20	N.D.
Oxoid	20-100	N.D.
Unilever	0-5	N.D.

N.D. = None detected

Table 3.2 Analysis of diet constituents for nitrosamines and mycotoxins

Constituent	Nitrosamines	Aflatoxin and Ochratoxin
Fish-meal	50-250 ppb DMN	N.D.
Soya-meal	N.D.	N.D.

N.D. = None detected

from consideration. The Spillers diet therefore offered the lowest nitrosamine content, but this was considered to be unacceptably high for this particular study. The protein source used in this diet was White Fish meal which has in the past been shown to contain nitrosamines (Koppang, 1974). Samples of this ingredient and of soya-bean meal, an alternative protein source, were analysed for nitrosamine and aflatoxin content. The results are shown in Table 3.2.

Substitution of the soya-bean meal for fish meal was then contemplated and, in conjunction with the research laboratories of Spillers, a diet was developed with this change in formulation. At the same time it was agreed that supplies of this diet would be maintained for the duration of the study.

Each fresh batch of diet delivered was analysed for content of the contaminants shown in Table 3.3. For each contaminant a maximum permitted concentration was defined. These concentrations were arrived at after analysis of a sample of the diet and a review of the likely effect of each of the contaminants. At no time during the study did any batch of diet contain more than the concentration of contaminants shown in the table.

The diet used was in the form of expanded pellets and was fed '<u>ad libitum</u>' from hoppers on each cage.

<u>Drinking Water</u>

To avoid any interference with the study, by nitrate or nitrite normally present in tap-water, distilled water was used throughout, both in the preparation of solutions

Table 3.3 Maximum permissible concentrations of dietary contaminants

Contaminant	Maximum permitted concentration
Nitrosamines	5 ppb
Nitrate	20 ppm
Nitrite	0.5 ppm
Aflatoxin & ochratoxin	5 ppb
Polycyclic aromatic hydrocarbons	20 ppb
DDT	250 ppb
Polychlorinated biphenyls	500 ppb
Mercury	7 ppb
Lead	2 ppm
BHT [added antioxidant]	10 ppm

and for administration to control animals.

Distilled water was produced by two Fi-stream 8 litre/hr stills. The output from these stills was fed into a 1000 litre capacity high-density polyethylene storage tank. Water was drawn from the tank when required, through food-grade PVC tubing, using a centrifugal pump. The tank was regularly drained and cleaned, using fresh distilled water, to prevent accumulation of any contamination.

ADMINISTRATION OF NITROSAMINES

Nitrosamine solutions were prepared weekly, and the concentration of nitrosamine in each solution was checked before use [Walters et al., 1970]. The volatile nature of the nitrosamines under study necessitated the development of procedures which minimised loss. The procedures for the preparation and dispensing of the solutions are described below.

To ensure that the animals received the intended solutions all drinking water bottles were drained each week prior to filling. These quantities of effete solutions and some excess preparation resulted in the production of considerable volumes of waste solutions. A method of neutralising this waste was developed [Gangolli et al., 1974].

Preparation of Solutions

The solutions were prepared in three stages of dilution. All preparation of solutions was carried out in a room within the animal unit that was specifically designated for this purpose. This room housed the stills and the storage-

-tank described previously, together with a refrigerator for storage of nitrosamines and a fume-cupboard for the preparation of solutions.

All mixing and dispensing operations were carried out in the fume-cupboard. Small volumes were dispensed with automatic pipettes using disposable tips, larger volumes with automatic dispensers. Solution preparation consisted of three stages, the first two of which were carried out using glass volumetric flasks. Mixing of solutions was by means of magnetic stirrers. Some of the containers used for the final solutions could not be accommodated in the fume-cupboard, and for these situations a system was employed which permitted handling of the solutions to continue in the fume-cupboard. This system of a funnel held in a retort-stand within the fume-cupboard, connected by tubing to a screw-on cap which fitted the container for the final solution. A second tube was led back from this cap to the fume-cupboard to provide an air-vent when the bottle was being filled.

The procedure used for the preparation of solutions is described below:

Stage 1

Stock solution in distilled water sufficient to last for 3-4 weeks were prepared at a concentration of 5.3% v/v. These solutions were stored in brown glass bottles at 4°C.

Stage 2

1 litre of a second dilution in distilled water was prepared each week, the concentration depended on the volumes of final solution that were required, [e.g. a final volume

of 8 litres required 10.0 ml to be diluted to 1 litre). At this stage the concentration was checked using a polarographic technique (Walters et al., 1970).

Stage 3

Volumes of second dilution as shown in Table 3.4 were dispensed into each final solution container and made up to volume with distilled water. The volumes of water added were measured using a flow meter calibrated in 10 ml divisions. These solutions were mixed, using a large magnetic stirrer, for ten minutes each.

Once all the final solutions had been prepared a small sample of each was taken for analysis.

Solutions were deemed acceptable for use if the analysed concentration was within 10% of that intended, with two exceptions. The dose levels, 1060 and 1590, and 2100 and 2600 were allowed a deviation of only 5% from the intended concentration, to prevent overlap.

All analyses were performed by the BIBRA Biological Chemistry department whose assistance is gratefully acknowledged. The polarograph technique (Walters et al., 1970) was used to analyse all solutions of greater than 1 ppm nominal concentration. The limit of detection for this technique did however, mean that it could not be used for solutions of nominal concentration less than 1 ppm. This problem was overcome in the following way:

Sodium chloride was added to the 'second dilution' referred to above to give a ratio of 20 gm NaCl to 0.53 ml nitrosamine. The ratio was checked at the time of analysis of this solution. The concentration of nitrosamine in the

Table 3.4 Volumes of second dilution to be taken for the preparation of the final solutions

Nominal concentration of final solution (ppb)	Volume of second dilution (ml)
33	0.5
66	1.0
130	2.0
260	4.0
530	8.0
1060	16.0
1580	24.0
2100	32.0
2600	40.0
3200	48.0
4200	64.0
5300	80.0
6300	96.0
8400	128.0
16900	256.0

low-dose solutions (those less than 1 ppm) was assumed to be correct if the appropriate concentration of sodium chloride was found to be present using an Atomic Absorption Spectrophotometer. At approximately 6-weekly intervals the accuracy of this procedure was checked by analysing the solutions of less than 1 ppm nominal concentration, using a gas-liquid chromatography technique (Alliston et al., 1972). Although this procedure could analyse solutions, at all concentrations used it was too slow for routine use.

Dispensing of Nitrosamine Solutions

The containers in which the final solutions were prepared were used to dispense these solutions into the animal drinking bottles. The bottles were filled in situ on the racks carrying the cages. The design of these dispensing containers is shown in Fig. 3.3(A).

Solutions were dispensed by applying positive pressure to the head space of the container. The air-pressure was developed by a pump fitted with a pressure release valve operating at 5 p.s.i. A fail-safe valve developed for the purpose (Fig. 3.3 (B)), and fitted to the output tubing enabled the bottles to be filled without spillage.

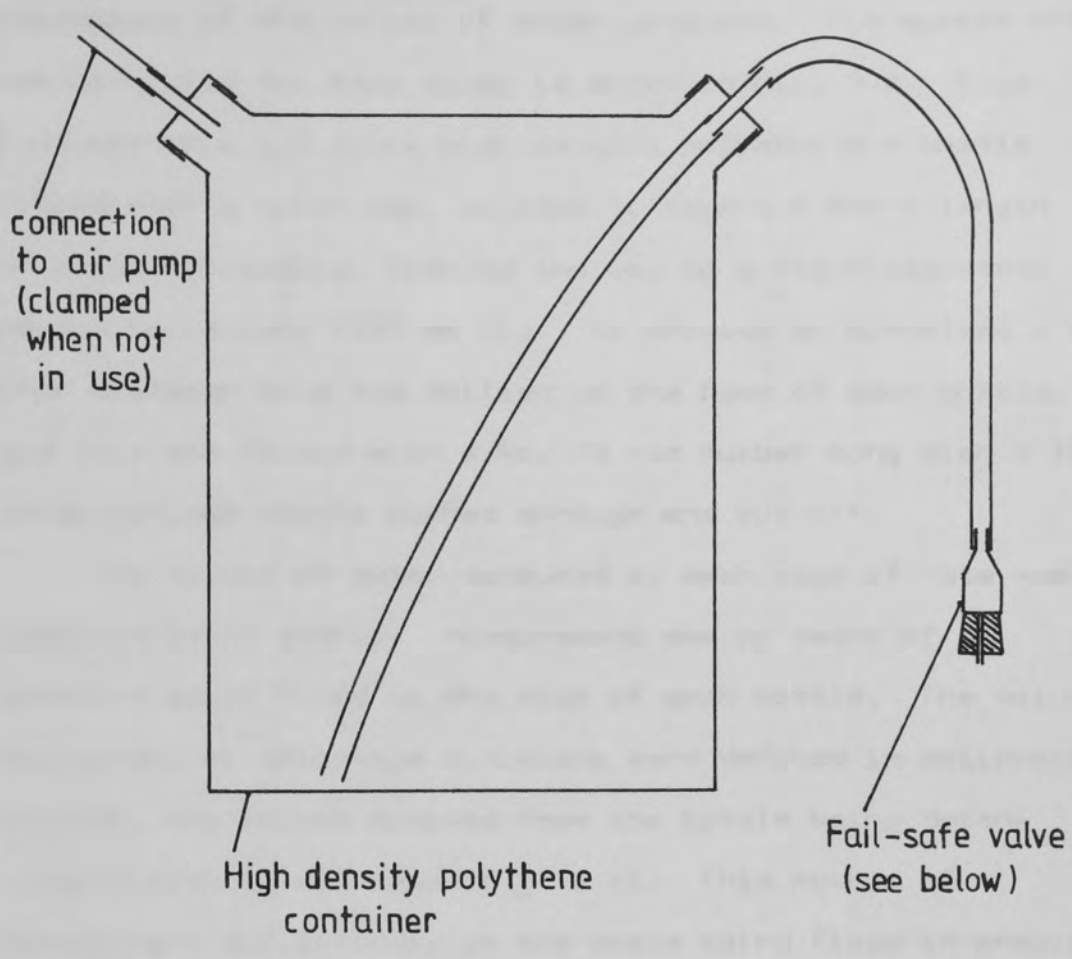
Weekly emptying of the drinking-bottles was carried out using a container similar to that used for dispensing but with negative pressure applied to the head-space.

Drinking-bottles

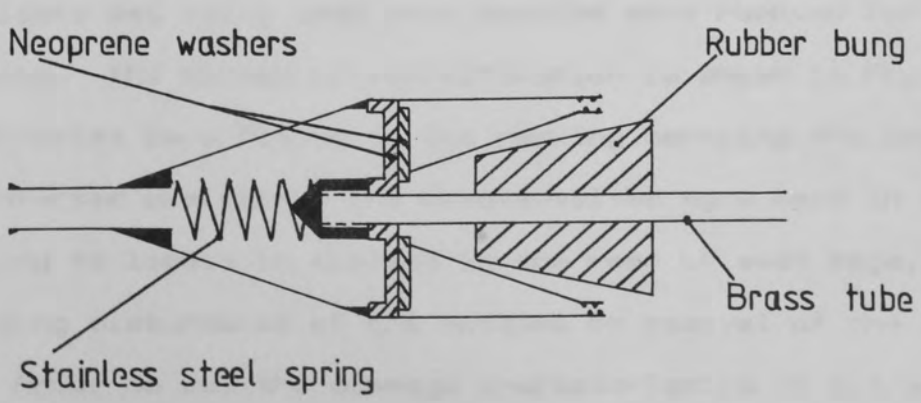
The volatile nature of the nitrosamines used in this study necessitated the development of an animal watering

FIGURE 3.3 DISPENSING SYSTEM FOR NITROSAMINE SOLUTIONS

[A] DISPENSING CONTAINER



[B] FAIL SAFE VALVE



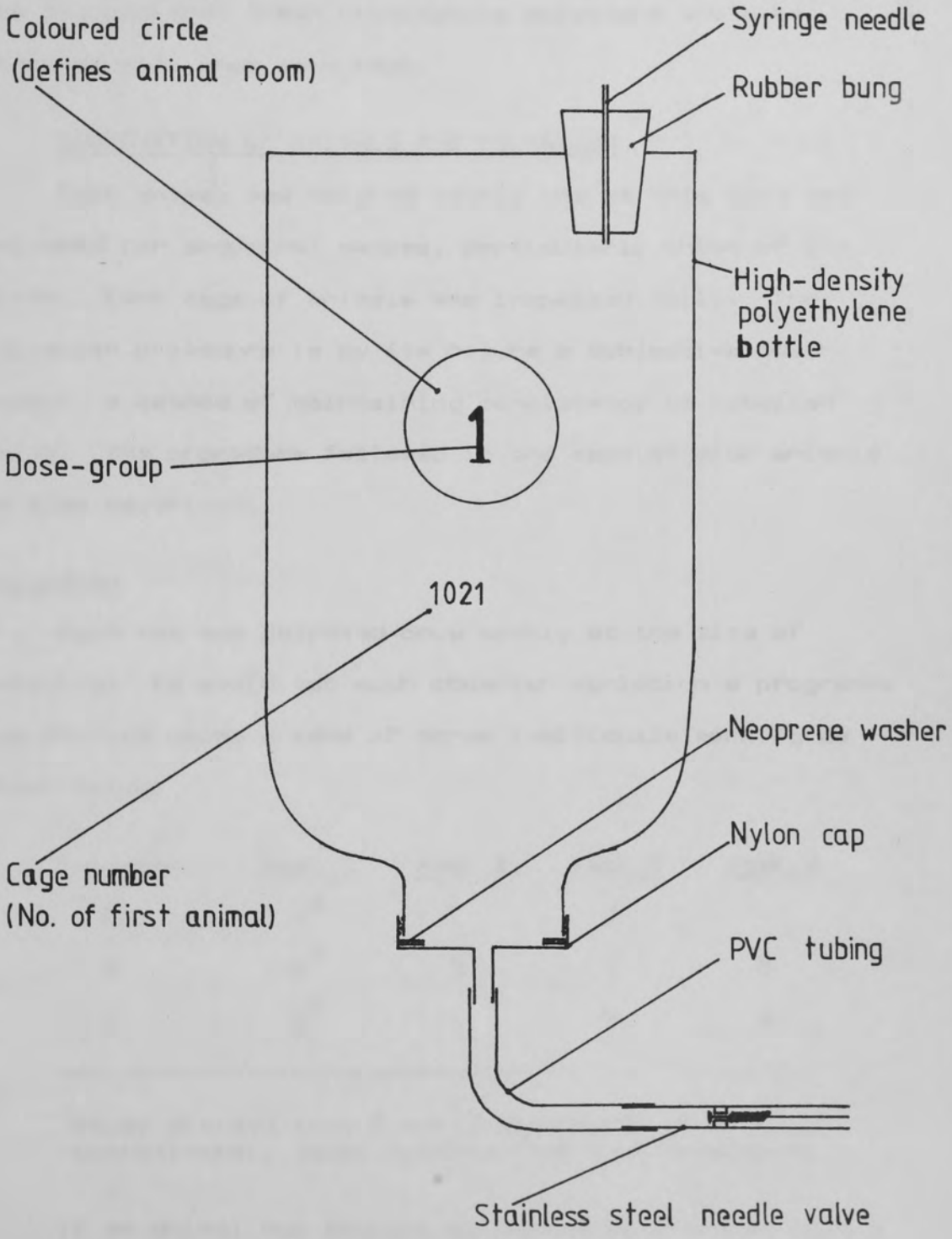
system which had minimal leakage but which also permitted assessment of the volume of water consumed. The system which was developed for this study is shown in Fig. 3.4. This consists of a 1.3 litre high-density polyethylene bottle fitted with a nylon cap, moulded to accept a short length of clear PVC tubing, linking the cap to a stainless-steel needle-valve (NKP TV25 Mk II). To provide an air-bleed a ½ inch diameter hole was drilled in the base of each bottle, and this was fitted with a No. 13 red rubber bung with a 19 gauge syringe needle pushed through and cut off.

The volume of water consumed by each cage of rats was measured twice weekly. Measurement was by means of an adhesive scale fixed to the side of each bottle. The volumes equivalent to the scale divisions were defined in calibration studies, the volume removed from the bottle being determinable with an accuracy of $\pm$ 10 ml. This method of measurement did not rely on the scale being fixed in precisely the same position on each bottle, as it simply required the difference between two readings from the parallel region of the bottle.

Each bottle for each cage was uniquely identified, a duplicate set being used when bottles were removed for washing. The method of identification is shown in Fig. 3.4. The bottles were fitted to the racking carrying the cages in an inverted position. The needle-valves were held in the racking to locate in eyelets in the rear of each cage, thus avoiding disturbance of the bottles on removal of the cages.

Prior to use the storage characteristics of nitrosamine solutions in these bottles was investigated. The results

FIGURE 3.4 DRINKING BOTTLE DESIGN AND IDENTIFICATION



demonstrated some variation but all within 10% of the intended concentration. On the basis of these results it was decided that fresh nitrosamine solutions would be prepared only once each week.

OBSERVATION OF ANIMALS AND PALPATION

Each animal was weighed weekly and at this time was palpated for abdominal masses, particularly those of the liver. Each cage of animals was inspected daily. The palpation procedure is by its nature a subjective observation, a method of maintaining consistency is detailed below. The procedure followed in the case of sick animals is also described.

Palpation

Each rat was palpated once weekly at the time of weighing. To avoid too much observer variation a programme was devised using a team of three individuals working as shown below:

Palpater	Week 1	Week 2	Week 3	Week 4
A	1+	2	3	1
B	2+	3	1	2
C	3+	1	2	3

+Study divided into 3 sub-groups containing approximately equal numbers from each treatment.

If an animal was thought by one of the team to have a palpable lump a second member of the team was called in to

confirm this observation. When two of the team were agreed that an animal had a palpable lump, that animal was taken immediately for autopsy.

Sick Rats

The animals found to be sick were monitored closely. When a sick rat reached a state where it could not reasonably be expected to survive a further 24 hours, it was taken for autopsy, together with any others found dead on the same day.

In a number of rats large subcutaneous masses developed. When these reached dimensions sufficient to seriously impede movement, or if ulceration occurred, these animals were taken for autopsy.

AUTOPSY PROCEDURES

Live animals brought to autopsy were killed by exsanguination from the aorta under deep barbiturate anaesthesia. To avoid any possible exchange of tissue samples or other form of confusion autopsies were carried out sequentially unless numbers (e.g. interim kills) dictated otherwise. The objective of the autopsy was defined clearly as the identification of the likely cause of sickness or death. Thus attention was concentrated on the macroscopic examination and description of all possible tissues, and routine sampling confined to only a few organs as described below.

Liver, kidneys, bladder, lungs, skull and oesophagus were taken from every animal together with any other tissue found to be abnormal. All tissues sampled were preserved in 10% buffered formalin.

All livers were examined for abnormality and the presence and position of all lesions was recorded. Each lobe of the liver was sampled after cutting into slices of approximately 5 mm thickness, and examination of the cut surfaces, two slices of each lobe were fixed separately in labelled tubes, to permit close correlation of histological and macroscopic findings.

Bladders were inflated with formalin before removal to ensure fixation of the epithelium. Lungs were also inflated in situ with formalin through the trachea, and then removed and fixed intact.

Oesophagi were removed complete with pharynx and the base of the tongue, opened up to reveal the mucosal surface and fixed intact on stiff card with the serosal surface against the card. The general position of any nodules and their size was recorded.

Skulls were fixed after removal of the brain and pituitary, and examination of the buccal cavity by cutting through one axis of the jaw. Ears were left in place so that identification could be checked if necessary.

Spinal cords were examined in situ by trimming off the dorsal part of the neural arches with bone-nibblers.

Tissues were stored in plastic pots and labelled using waterproof marker on non-peelable labels.

HISTOPATHOLOGY

All tissues collected at autopsy were examined after fixation, and, with reference to the autopsy report, appropriate samples were trimmed. All samples were processed

using an automated wax-embedding technique and stained with haematoxylin and eosin.

Microscopic examination of the sections was carried out with reference to the autopsy report, every attempt being made to identify the histological nature of the lesions seen at autopsy.

Where tumours were seen each was identified according to the degree of differentiation, the probable cell-type of origin, presence or absence of invasion and metastasis and the general appearance of the cells.

SAFETY

As large volumes of nitrosamine solutions were involved in this study, safety was a major consideration.

The unit in which the study was carried out was totally devoted to this function and contained a room for the preparation of solutions and its own cage-wash facilities.

The risk attached to handling nitrosamine solutions can conveniently be divided into two areas, skin contact and inhalation. These two problems are considered separately below.

Skin contact risk

Protective clothing was provided for every member of staff employed in the unit, consisting of rubber boots, a gown, a hat, a face-visor and thick rubber gloves. This clothing was worn when all operations were being performed in the animal rooms; apart from handling the rats, when surgical gloves were worn.

Any member of staff involved in the preparation of

solutions was required in addition, to wear a full-face gas-mask with an appropriate cannister attached.

In addition to the protective clothing each member of staff received a set of rules and instructions governing the wearing of the clothing and defining the procedure to be followed in the event of accidental spillage or contact with the nitrosamine solutions.

Occasionally spillages did occur and in these instances the animal room involved was immediately evacuated. The spillage was cleared by an individual in full protective clothing (including the respirator). The method of clearing the spillage was to soak up the solution using paper tissue; this was then sealed in a polythene bag which was sealed in a second bag and taken for immediate incineration. The room was then locked and no access allowed for at least one hour (15 changes of air). On no occasion did any staff report skin contact with the nitrosamine solutions.

Inhalation risk

Perhaps the highest risk area, as for skin contact, was the preparation room; the only area in the study where solutions containing more than 17 ppm nitrosamine were handled. This risk was considered adequately met by the use of respirators in this area.

Throughout the rest of the facility the systems for dispensing the solutions and the drinking bottles attached to the cages were designed to minimuse risk in this respect. Analysis of the solutions demonstrated that there was no

detectable loss of nitrosamine from either the drinking or dispensing bottles. Coupled with the flow-rate of air in the animal rooms, the risk was regarded as minimal.

A major source of risk in any situation is under-staffing, which tends to lead to short-cuts in procedures. Adequate staffing levels were ensured at all times during this study. Similarly no individual was permitted to work alone in the unit at any time.

Procedures were developed for the disposal of all solid waste by incineration, as was a process for the neutralisation of waste solutions (page 41).

CHAPTER FOUR

RESULTS

Intake of Nitrosamine

Samples of the body-weight and water-intake were analysed to determine the mg/kg/day intakes of both nitrosamines. As neither body weight nor water intake appeared to be affected by treatment the intakes were based on a mean figure for water intake derived from all groups. The result of these calculations is shown in table 4.1.

Mortality

Deaths occurred in the highest dose-groups of both nitrosamines after approximately 6 months of treatment. Subsequent mortality followed a dose-related pattern, with deaths occurring earlier at the higher doses (tables 4.2-4.5). In animals receiving less than 1056 ppb DEN or 1584 ppb DMN the mortality rate at all times was very similar to that of controls. Plots of cumulative mortality for both sexes of controls allow a median lifespan to be derived. This is estimated to be 920 days for males and 837 days for females (figures 4.1-4.2).

Tumour incidences

The majority of animals had at least one tumour at death and the incidence of all tumours found in each dose-group, classified by site is given in tables 4.6 to 4.9. Of the control animals 79% of males and 94% of females had a tumour at death.

A considerable number of tumours showed variation in incidence related to the dose of nitrosamine. The incidence of tumours of pituitary, uterus and mammary tissue was significantly reduced, compared with controls, in the higher

Table 4.1 Calculated mean mg/kg/day intakes of DEN and DMN by animals receiving these nitrosamines in their drinking water

Concentration (ppm)	mg/kg/day Males	mg/kg/day Females
0.033	0.002	0.003
0.066	0.004	0.006
0.132	0.008	0.011
0.264	0.017	0.02
0.528	0.03	0.05
1.06	0.07	0.09
1.58	0.10	0.14
2.11	0.14	0.18
2.64	0.17	0.23
3.17	0.20	0.28
4.22	0.27	0.37
5.28	0.34	0.46
6.34	0.41	0.55
8.45	0.54	0.73
16.9	1.08	1.47

Table 4.2 Cumulative mortality [%] of male rats receiving DEN in the drinking water

Concentration [ppm]	Accumulated % mortality at day						
	200	400	600	800	1000	1200	1400
0 [control]	2	4	7	29	71	99	100
0.033	0	0	6	29	69	100	
0.066	0	2	4	19	56	100	
0.132	0	2	6	29	63	100	
0.264	8	10	16	33	73	100	
0.528	2	2	8	18	62	100	
1.06	2	4	10	29	83	100	
1.58	0	0	6	46	96	100	
2.11	4	8	26	89	100		
2.64	0	0	29	95	100		
3.17	0	0	31	100			
4.22	0	8	81	100			
5.28	2	21	94	100			
6.34	0	10	100				
8.45	0	54	100				
16.9	2	98	100				

Table 4.3 Cumulative mortality [%] of male rats receiving DMN in the drinking water

Concentration [ppm]	Accumulated % mortality at day						
	200	400	600	800	1000	1200	1400
0 (control)	2	4	7	29	70	99	100
0.033	0	2	4	23	65	100	
0.066	0	0	2	29	71	100	
0.132	0	2	11	27	63	100	
0.264	0	2	2	33	71	98	100
0.528	0	0	6	21	61	98	100
1.06	0	0	11	36	79	98	100
1.58	0	2	4	36	81	100	
2.11	0	0	8	31	73	100	
2.64	0	4	17	65	94	100	
3.17	0	2	21	56	100		
4.22	2	11	27	75	98	100	
5.28	0	6	71	100			
6.34	0	11	75	100			
8.45	2	29	100				
16.9	25	100					

Table 4.4 Cumulative mortality (%) of female rats receiving DEN in the drinking water

Concentration (ppm)	Accumulated % mortality at day						
	200	400	600	800	1000	1200	1400
0 (control)	0	2	9	40	83	99	100
0.033	0	0	8	33	86	100	
0.066	0	0	8	48	86	100	
0.132	0	0	11	46	94	100	
0.264	0	0	6	27	81	98	100
0.528	0	4	15	56	98	100	
1.06	0	0	8	63	100		
1.58	0	2	21	92	100		
2.11	0	0	65	98	100		
2.64	0	2	86	100			
3.17	0	6	100				
4.22	0	38	100				
5.28	0	54	100				
6.34	0	90	100				
8.45	0	98	100				
16.9	12	100					

Table 4.5 Cumulative mortality (%) of female rats receiving DMN in the drinking water

Concentration (ppm)	Accumulated % mortality at day						
	200	400	600	800	1000	1200	1400
0 (control)	0	2	9	40	83	99	100
0.033	2	4	6	38	71	100	
0.066	2	2	13	48	79	98	100
0.132	2	6	11	50	83	100	
0.264	0	0	2	42	83	100	
0.528	0	2	15	52	94	98	100
1.06	2	2	11	44	78	100	
1.58	0	0	21	77	96	100	
2.11	0	2	25	73	100		
2.64	0	4	48	98	100		
3.17	0	4	65	98	100		
4.22	0	10	88	100			
5.28	0	29	100				
6.34	0	27	100				
8.45	13	88	100				
16.9	56	100					

Table 4.6 Incidence of tumours in male rats receiving DEN in the drinking water

Tumour site	Tumour incidence (%) in males receiving (ppb DEN)															
	0	33	66	132	264	528	1060	1580	2110	2640	3170	4220	5280	6340	8450	16900
Liver	8	10	8	21	6	21	40	58	40	60	54	65	58	67	58	81
Oesophagus	0	0	0	0	0	6	33	65	73	83	92	75	91	79	83	88
Nasopharynx	0	0	0	0	0	0	4	2	2	2	0	2	2	0	0	0
Kidney	1	0	6	0	0	0	0	0	2	0	0	0	0	0	0	0
Bladder	2	0	2	0	0	0	2	2	0	0	2	0	2	0	0	0
Stomach	1	0	2	0	0	0	0	0	0	0	0	0	0	4	0	0
Small intestine	3	2	2	4	2	4	2	0	0	0	0	0	0	0	0	0
Caecum	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Colon	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lung	0	0	2	0	2	0	0	0	0	0	0	0	0	0	0	0
Heart	5	6	4	2	4	4	2	0	0	0	0	0	0	0	0	0
Thymus	4	2	0	4	2	0	0	0	2	2	0	0	0	0	0	0
Trachea	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0
Thyroid	9	13	4	8	10	13	13	2	4	0	0	0	0	0	0	0
Salivary gland	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0
Mammary tissue	2	0	0	0	2	0	0	0	0	0	0	2	0	0	0	0
S/c tissue	10	19	8	4	17	2	21	13	2	0	0	0	0	0	2	0

Table 4.6 Continued

Tumour site	Tumour incidence (%) in males receiving (ppb DEN)															
	0	33	66	132	264	528	1060	1580	2110	2640	3170	4220	5280	6340	8450	16900
Skin	1	2	4	0	0	10	0	2	4	0	0	2	0	0	0	0
Harderian gland	0	0	2	0	0	0	0	2	0	0	0	2	0	0	0	0
Brain	7	4	10	8	4	8	0	4	4	2	0	0	0	0	0	0
Tongue	1	0	0	0	0	0	2	2	0	0	0	0	0	0	0	0
Pituitary	26	23	19	23	21	31	23	6	6	4	4	2	0	0	0	0
Adrenal	4	4	0	2	0	6	0	0	0	0	0	0	0	0	0	0
Pancreas	4	0	0	0	4	2	0	0	0	0	0	0	0	0	0	0
Spleen/Lymph nodes	7	10	10	8	2	8	4	2	6	2	0	2	0	0	0	0
Testes	7	10	10	8	8	17	8	2	2	0	0	0	0	0	0	0
Seminal Vesicle	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0
Prostate	0	0	2	0	0	0	2	0	0	0	0	0	0	0	0	0
Ureter	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0
Bone-marrow	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0
Bone	1	2	0	2	2	0	0	2	0	0	0	0	0	0	0	0
Skeletal muscle	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0
Reticulo-endothelial system	4	0	0	2	0	0	8	0	0	0	0	0	2	0	0	0

Table 4.6 Continued

Tumour site	Tumour incidence (%) in males receiving (ppb DEN)															
	0	33	66	132	264	528	1060	1580	2110	2640	3170	4220	5280	6340	8450	16900
Connective tissue	1	2	2	2	0	0	0	0	0	0	0	0	0	0	0	0
Lower jaw	3	4	0	4	6	8	4	0	2	0	2	0	0	0	0	0
Spinal cord	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Peripheral and cranial nerves	1	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0
Peritoneum	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Feet	0	2	0	2	0	0	0	0	0	0	0	0	0	0	0	0
Adamantinoma	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Incidences shown are for treated groups of 48 rats and control group (0 ppb DEN) of 192 rats receiving the appropriate concentration of DEN in their drinking water throughout their lives.

Table 4.7 Incidence of tumours in male rats receiving DMN in the drinking water

Tumour site	Tumour incidence (%) in males receiving (ppb DMN)															
	0	33	66	132	264	528	1060	1580	2110	2640	3170	4220	5280	6340	8450	16900
Liver	8	19	21	13	17	15	25	31	48	69	79	79	92	98	100	92
Kidney	1	4	0	0	4	4	0	0	0	0	0	0	0	0	0	0
Bladder	2	0	0	0	0	0	2	2	2	0	2	0	0	0	2	0
Stomach	1	0	2	2	0	0	0	0	0	0	2	2	2	0	0	0
Small intestine	3	0	0	4	8	0	4	0	0	2	2	2	0	0	0	0
Caecum	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Colon	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lung	0	0	0	0	2	0	2	2	2	0	0	0	0	0	0	0
Heart	5	6	8	4	6	2	2	4	0	4	0	0	0	0	0	0
Thymus	4	0	2	2	0	0	2	0	2	0	2	2	0	0	0	0
Thyroid	9	17	10	10	17	19	15	2	8	6	6	2	0	0	0	0
Salivary gland	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Mammary tissue	2	0	0	0	0	2	0	0	4	0	0	0	0	0	0	0
S/c tissue	10	15	10	13	8	15	10	10	10	4	4	4	0	2	0	0

Table 4.7 Continued

Tumour site	Tumour incidence (%) in males receiving (ppb DMN)															
	0	33	66	132	264	528	1060	1580	2110	2640	3170	4220	5280	6340	8450	16900
Skin	1	0	0	0	2	0	0	2	4	0	6	0	0	0	0	0
Harderian gland	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0
Brain	7	8	2	4	4	0	6	4	4	8	4	0	0	0	0	0
Tongue	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pituitary	26	33	29	29	23	40	25	35	25	23	13	4	4	0	0	0
Adrenal	4	2	2	4	6	0	2	4	2	2	4	0	0	0	0	0
Pancreas	4	2	2	6	0	4	4	2	2	0	0	2	0	0	0	0
Spleen/Lymph nodes	7	2	4	2	0	8	4	6	2	2	0	8	0	0	0	0
Testes	7	0	8	4	2	10	4	8	6	6	0	0	0	0	0	0
Prostate	0	0	2	0	0	2	2	0	2	2	2	0	2	0	0	0
Cowpers complex	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0
Bone-marrow	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0
Bone	1	0	0	4	0	2	2	2	0	2	0	0	0	0	0	0
Skeletal muscle	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0
Reticulo-endothelial system	4	0	6	2	2	0	0	0	0	0	0	2	0	0	0	0

Table 4.7 Continued

Tumour site	Tumour incidence (%) in males receiving (ppb DMN)															
	0	33	66	132	264	528	1060	1580	2110	2640	3170	4220	5280	6340	8450	16900
Connective tissue	1	0	0	2	0	0	0	0	2	0	0	2	0	0	0	0
Lower jaw	3	4	6	6	2	2	2	6	4	4	0	0	0	0	0	0
Spinal cord	1	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Ear	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0
Cranial nerves	1	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0
Peritoneum	1	0	0	0	0	0	2	0	2	0	0	0	0	0	0	0
Feet	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0
Site uncertain	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0

Incidences shown are for treated groups of 48 rats and a control group (0 ppb DMN) of 192 rats receiving the appropriate concentration of DMN in their drinking water throughout their lives.

Table 4.8 Incidence of tumours in female rats receiving DEN in the drinking water

Tumour site	Tumour incidence (%) in females receiving (ppb DEN)															
	0	33	66	132	264	528	1060	1580	2110	2640	3170	4220	5280	6340	8450	16900
Liver	14	21	19	10	31	40	77	88	87	92	94	96	79	92	96	93
Oesophagus	0	0	0	0	0	6	35	42	52	48	71	63	65	77	69	62
Kidney	1	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0
Bladder	1	0	0	0	4	0	0	0	0	0	0	0	0	0	0	0
Stomach	2	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0
Small intestine	1	0	2	2	0	0	0	0	0	0	0	0	0	0	0	0
Caecum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Colon	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Rectum	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lung	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Heart	2	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0
Thymus	9	19	13	10	2	6	4	4	0	0	0	0	0	0	0	0
Trachea	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Thyroid	6	8	6	6	2	6	0	0	0	0	0	0	0	0	0	0
Salivary gland	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Table 4.8 Continued

Tumour site	Tumour incidence (%) in females receiving (ppb DEN)															
	0	33	66	132	264	528	1060	1580	2110	2640	3170	4220	5280	6340	8450	16900
Mammary tissue	12	19	13	10	21	8	6	2	0	0	0	0	0	2	0	0
S/c tissue	9	2	15	8	13	2	8	2	0	0	0	0	0	0	0	0
Skin	0	0	2	0	4	0	0	0	0	0	0	0	0	0	0	0
Eye	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Harderian gland	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Brain	1	4	0	0	2	2	2	4	0	0	0	0	0	0	0	0
Tongue	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pituitary	38	41	52	33	46	46	29	13	2	6	0	0	0	0	0	0
Adrenal	1	2	0	2	2	6	0	4	0	0	0	0	0	0	0	0
Pancreas	0	2	0	0	2	0	0	0	0	0	0	0	0	0	0	0
Spleen/lymph nodes	4	0	6	0	0	2	0	0	0	0	0	0	0	0	0	0
Ovaries	2	0	0	2	2	2	0	0	0	0	0	0	0	0	0	0
Uterus	40	35	33	38	42	50	31	17	11	11	2	0	0	2	0	0
Ureter	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bone-marrow	0	0	0	0	2	0	2	0	0	0	0	0	0	0	0	0
Bone	1	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0
Skeletal muscle	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Table 4.8 Continued

Tumour site	Tumour incidence (%) in females receiving (ppb DEN)															
	0	33	66	132	264	528	1060	1580	2110	2640	3170	4220	5280	6340	8450	16900
Diaphragm	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Adipose tissue	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Reticulo-endothelial system	2	2	0	10	2	0	2	0	0	0	0	0	0	0	0	0
Connective tissue	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lower jaw	4	2	0	0	4	4	2	0	0	0	0	0	0	0	0	0
Spinal cord	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Ear	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cranial nerves	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Peritoneum	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0
Feet	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cartilage	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Adamantinoma	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Blood vessels	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Site uncertain	1	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0

Incidences shown are for treated groups of 48 rats and a control group (0 ppb DEN) of 192 rats receiving the appropriate concentration of nitrosamine in their drinking-water throughout their lives.

Table 4.9 Incidence of tumours in female rats receiving DMN in the drinking water

Tumour site	Tumour incidence (%) in females receiving (ppb DMN)															
	0	33	66	132	264	528	1060	1580	2110	2640	3170	4220	5280	6340	8450	16900
Liver	14	25	21	19	27	29	44	79	81	96	98	100	96	98	92	85
Nasal cavity	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0
Kidney	1	0	2	0	0	0	4	0	2	0	0	0	0	0	0	0
Bladder	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Stomach	2	2	2	0	0	2	0	0	0	0	0	0	0	0	0	0
Small intestine	1	0	2	0	2	0	0	0	0	0	0	0	0	0	0	0
Rectum	1	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0
Lung	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0
Heart	2	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Thymus	9	23	10	6	15	17	10	6	4	2	0	0	0	0	0	0
Thyroid	6	2	4	2	0	6	4	0	0	0	0	0	0	0	0	0
Salivary gland	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Mammary tissue	12	8	13	17	15	8	13	2	13	2	4	0	0	0	0	0
S/C tissue	9	8	13	8	13	6	10	8	4	0	0	2	0	0	0	0
Skin	0	2	2	0	0	0	0	0	0	0	0	0	0	0	0	0
Brain	1	4	2	2	4	4	2	0	4	0	0	0	0	0	0	0
Pituitary	38	48	27	46	38	35	35	21	15	19	6	4	2	0	0	0

Table 4.9 Continued

Tumour site	Tumour incidence (%) in females receiving (ppb DMN)															
	0	33	66	132	264	528	1060	1580	2110	2640	3170	4220	5280	6340	8450	16900
Adrenal	1	2	2	2	0	0	2	0	2	2	2	0	0	0	0	0
Pancreas	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Spleen/lymph nodes	4	2	2	2	2	6	13	2	0	2	0	0	0	0	0	0
Ovaries	2	4	4	2	2	0	4	0	0	0	0	0	0	0	0	0
Uterus	40	35	38	54	38	31	31	31	17	13	10	4	6	2	0	0
Bone	1	2	0	0	2	0	2	4	0	0	2	0	0	0	0	0
Skeletal muscle	1	0	0	2	0	0	0	0	2	0	0	0	0	0	0	0
Reticulo-endothelial system	2	0	2	2	0	2	2	0	0	0	0	0	0	2	0	2
Connective tissue	1	0	0	0	0	2	0	0	0	2	0	0	0	0	0	0
Lower jaw	4	2	2	8	8	0	4	0	0	0	0	0	0	2	0	0
Peritoneum	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0
Cartilage	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Site uncertain	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Incidences shown are for treated groups of 48 rats and a control group (0 ppb DMN) of 192 rats receiving the appropriate concentration of DMN in their drinking-water throughout their lives.

Figure 4.1 This figure shows the cumulative mortality of 192 untreated male rats maintained as controls for animals receiving either DMN or DEN in the drinking water.

Fig. 4.1 MORTALITY — MALE CONTROL

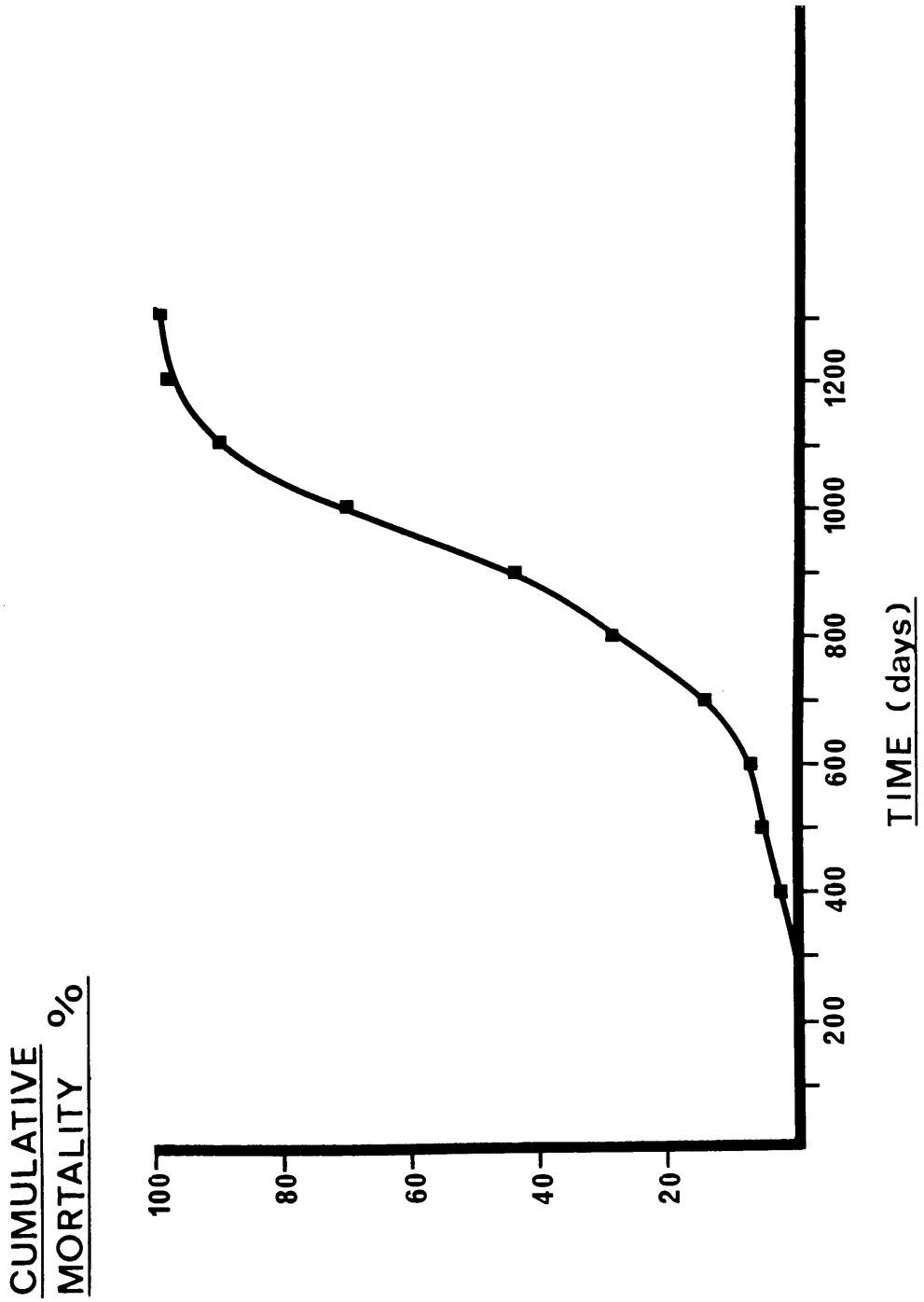
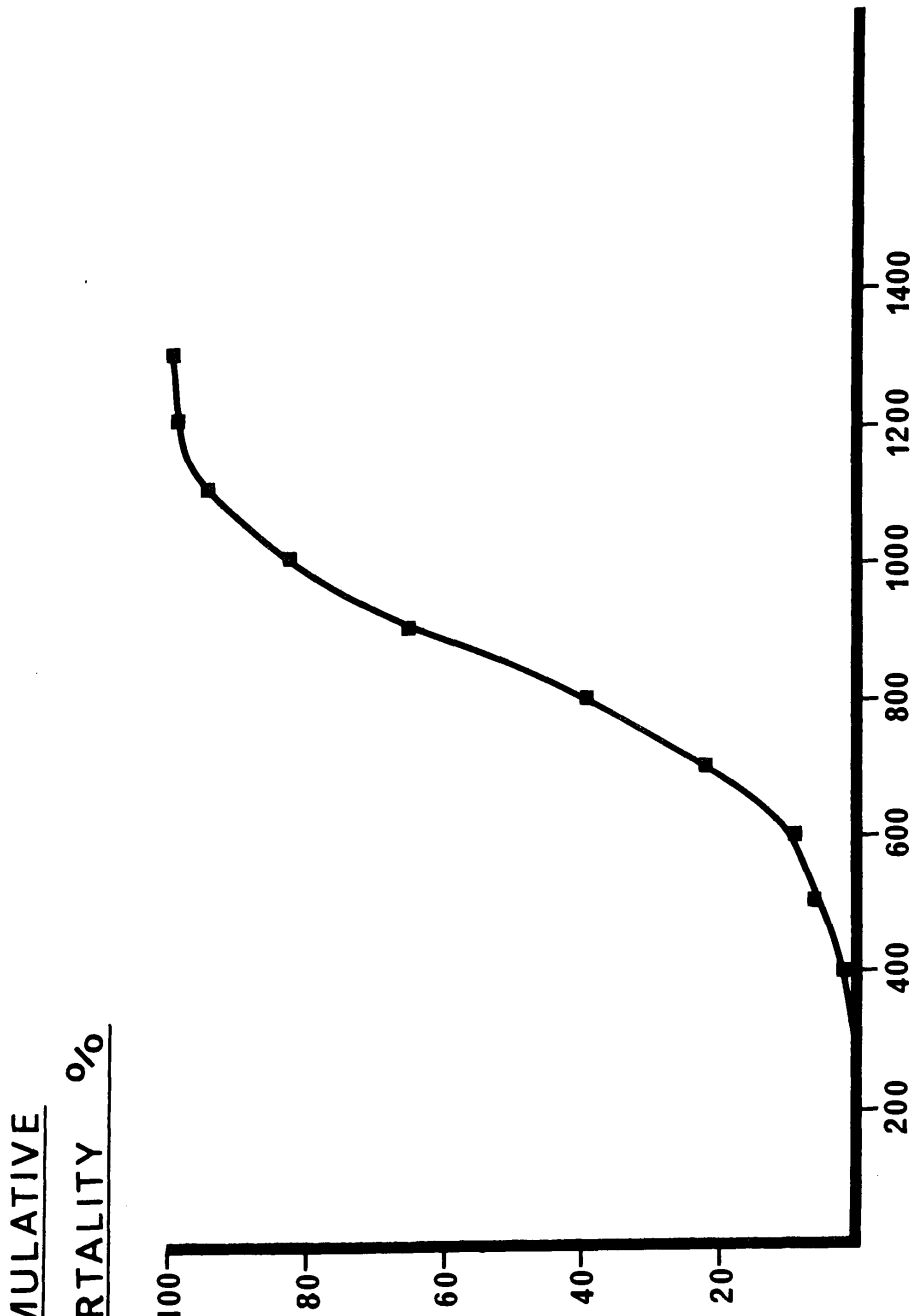


Figure 4.2 This figure shows the cumulative mortality of 192 untreated female rats maintained as controls for animals receiving either DMN or DEN in their drinking water.

Fig.4.2 MORTALITY — FEMALE CONTROL



dose-groups of both nitrosamines. This effect, along with the reduced incidence of several other tumour types, is an effect of treatment in that the animals died of treatment--related tumours before the normal time of appearance of these spontaneous tumours. It clearly only represents a protective effect of treatment insofar as early death protects an individual from the hazards of growing old. The incidence of a small number of tumour types was increased in treated animals compared with controls. For diethyl-nitrosamine, treatment was related to increased incidence of tumours of:

Liver

Oesophagus

Nasal epithelium.

For dimethylnitrosamine the only site showing a treatment--related increase in incidence of tumours was the liver.

Treatment-related tumours

The liver is a target-organ common to both nitrosamines. The full spectrum of change was seen in hepatocytes, with both DEN and DMN, ranging from nodular hyperplasia to totally anaplastic carcinoma. Hepatocellular tumours were common with both nitrosamines but were seen slightly more frequently with DEN. Other tumour types occurred originating from every cell-type present in normal liver. Haemangiomatous tumours were the next most common being seen more frequently following DMN treatment. While benign tumours of the biliary epithelium occurred with both nitrosamines only two malignant tumours of this origin were seen in the whole

study. The biliary cystadenomas were the commonest type of tumour in the mid-dose groups of DMN. Finally, the Kupffer cells gave rise to a small number of tumours following treatment with either nitrosamine. The incidences of the different types of liver tumours are shown in tables 4.10 to 4.13. Figures 4.3 to 4.6 illustrate the macroscopic appearance of the various tumour types.

In diethylnitrosamine-treated animals tumours of the oesophagus and nasopharynx showed treatment-related incidences. The oesophageal tumours originated in the squamous epithelium and were frequently multiple, being clearly visible at autopsy as papillary growths protruding into the oesophageal lumen. The commonest site for such nodules was at the upper end of the oesophagus in the epiglottis region, where a small nodule rapidly caused deterioration in the health of the animal. Nodules were also seen in these animals extending into the pharyngeal region, up to the base of the tongue.

Malignant oesophageal tumours were frequently encountered in DEN-treated animals, diagnosed by their invasive properties, however, only one such tumour in the whole study showed evidence of metastasis. The response to treatment with DEN was perhaps more clearly seen with oesophageal tumours than with those of the liver, due to the total absence of spontaneous tumours of this region in almost 2000 DMN-treated and control animals. A typical oesophageal lesion is shown in the photograph in figure 4.7.

Tumours of the nasopharynx were not detected macroscopically, however a few were seen on microscopic examination

Table 4.10 Incidences of liver tumours in male rats receiving DEN in the drinking water

Tumour type	Tumour incidence (%) in males receiving (ppb DEN)															
	0	33	66	132	264	528	1060	1580	2110	2640	3170	4220	5280	6340	8450	16900
Hep. nodules	2	6	4	2	2	6	10	21	15	21	8	8	15	17	6	21
Hep. adenoma	3	2	4	4	0	0	4	8	6	6	4	6	2	0	0	0
Hep.carcinoma	1	2	0	2	0	10	15	42	15	35	38	40	46	52	50	75
Haemangioma	0	0	0	0	0	0	6	0	2	0	0	0	0	0	0	0
Haemangio-sarcoma	1	0	0	6	2	6	0	6	6	4	10	10	0	4	0	0
Biliary cystadenoma	1	4	0	2	2	0	8	4	2	4	6	6	4	0	0	0
Bile-duct carcinoma	0	0	0	0	0	0	2	2	0	0	0	0	0	0	0	0
K-cell tumour	0	0	0	2	0	2	0	2	0	0	2	2	0	6	2	0
Type uncertain	0	0	0	2	0	0	0	0	4	0	0	2	2	0	2	0

Table 4.11 Incidences of liver tumours in female rats receiving DEN in the drinking water

Tumour type	Tumour incidence (%) in females receiving (ppb DEN)															
	0	33	66	132	264	528	1060	1580	2110	2640	3170	4220	5280	6340	8450	16900
Hep. nodules	7	6	13	8	15	25	33	35	44	40	63	54	58	67	48	45
Hep. adenoma	5	6	2	4	0	17	17	13	20	8	15	6	6	4	2	2
Hep.carcinoma	1	4	4	2	4	6	46	71	61	85	77	81	75	81	94	88
Haemangioma	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0
Haemangio-sarcoma	3	0	0	0	2	2	4	0	0	0	0	0	0	0	2	0
Biliary cysta-denoma	2	4	0	2	4	2	6	6	4	6	4	2	2	0	2	5
Bile-duct carcinoma	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
K-cell tumour	0	0	0	0	2	0	2	0	0	0	0	2	0	0	0	0
Type uncertain	1	0	2	0	4	0	0	0	2	0	4	2	0	0	0	2

Table 4.12 Incidences of liver tumours in male rats receiving DMN in the drinking water

Tumour type	Tumour incidence (%) in males receiving (ppb DMN)															
	0	33	66	132	264	528	1060	1580	2110	2640	3170	4220	5280	6340	8450	16900
Hep. nodules	2	13	10	2	2	4	2	6	8	10	10	10	6	2	6	8
Hep. adenoma	3	6	4	0	6	2	0	6	2	0	2	2	0	0	0	0
Hep. carcinoma	1	2	2	4	2	6	10	13	10	25	29	33	58	60	77	88
Haemangioma	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0
Haemangio-sarcoma	1	0	0	2	4	0	2	2	13	13	29	21	6	15	6	6
Biliary cystadenoma	1	4	4	4	4	2	2	8	13	23	27	25	29	40	29	4
Bile-duct carcinoma	0	0	0	0	0	0	0	0	0	2	0	2	0	0	0	0
K-cell tumour	0	0	2	0	0	0	4	0	0	6	0	0	4	0	0	0
Type uncertain	0	0	0	0	0	0	4	0	6	0	2	0	0	0	2	0

Table 4.13 Incidences of liver tumours in female rats receiving DMN in the drinking water

Tumour type	Tumour incidence (%) in females receiving (ppb DMN)															
	0	33	66	132	264	528	1060	1580	2110	2640	3170	4220	5280	6340	8450	16900
Hep. nodules	7	15	8	6	13	17	17	13	15	19	13	15	19	23	21	17
Hep. adenoma	5	4	4	4	4	2	6	4	2	6	0	0	0	0	0	0
Hep.carcinoma	1	0	0	4	4	10	6	10	10	8	13	15	25	38	69	73
Haemangioma	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Haemangio-sarcoma	1	2	0	2	0	2	4	2	6	2	6	4	0	0	6	10
Biliary cystadenoma	2	2	8	0	6	10	15	71	69	83	92	90	85	69	33	8
Bile-duct carcinoma	1	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0
K-cell tumour	0	0	0	0	0	2	2	0	2	0	0	0	0	0	0	0
Type uncertain	1	6	0	4	6	0	2	0	0	0	0	0	0	2	2	0

Figure 4.3 Solid lobulated multifocal tumour. Diagnosed microscopically as a well-differentiated hepatocellular carcinoma. Hyperplastic nodules also present. [8448 ppb DEN ♂ 4239]

Figure 4.4 Part cystic, part solid, multifocal tumour with very varied appearance. Diagnosed microscopically as two distinct tumours, hepatocellular carcinoma and haemangiosarcoma. [4224 ppb DMN ♂ 3529]

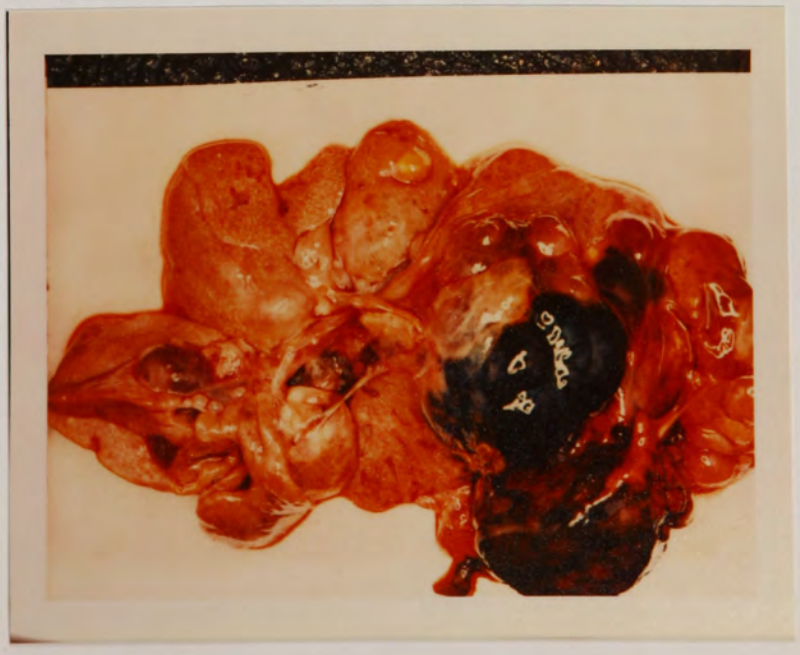


Figure 4.5 Totally cystic tumour, all cysts clear and transparent. Diagnosed microscopically as a biliary cystadenoma. [6336 ppb DMN ♀ 3797]

Figure 4.6 Only slightly raised nodules on this liver but with the whole surface having a fine gravel type appearance. Diagnosed microscopically as a Kupffer-cell sarcoma. A haemangiosarcoma was also diagnosed. [3168 ppb DEN ♂ 1587].

Figure 4.7 Oesophagus showing 4 separate large nodules, each indicated [◀] including one at the base of the tongue and another in a position where the epiglottis was obstructed (magnification x 2). Diagnosed microscopically as papillomas and squamous cell carcinoma.

of the skulls. Again, no tumours of this type were seen in the controls.

There was no other clear association between treatment and incidence of tumours at a specific site, however a complete time-related analysis was not carried out for all tumour types.

Relationship between dose and tumour incidence

As DEN causes tumours at two sites [liver and oesophagus], the first examination of the data was to check if the preferred site changed with dose. A plot of incidence of liver and oesophageal tumours against dose showed that the incidence of tumours at the two sites followed a similar pattern in relation to dose [figures 4.8 and 4.9]. There was a noticeable sex difference in that males seemed more likely to develop oesophageal tumours than females. It was concluded that the most useful analysis of the data from this study would be based on comparison of the total incidence of treatment-related tumours at different doses. Tables 4.14 and 4.15 show the incidences of the tumours at each site and the incidence of animals with a tumour at both sites and the incidence of animals with a tumour at one or the other site. The incidences of fatal tumours will be discussed in a later section.

Figure 4.10 shows the incidence of treatment-related tumours plotted against dose of DEN. Over much of the dose-range this plot runs close to 100% incidence and parallel to the dose axis. At doses below 0.2 mg/kg/day incidence is reduced in a dose-related manner. The incidence

Table 4.14 Incidence of treatment-related findings in male rats maintained on drinking water containing DEN

	No. of male rats with finding in group receiving (ppb DEN)															
	0	33	66	132	246	528	1060	1580	2110	2640	3170	4220	5280	6340	8450	16900
No. of rats	192	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48
Liver tumour	11	4	0	9	2	7	15	25	16	24	29	29	24	29	26	37
Oesophageal tumour	0	0	0	0	0	3	16	29	35	40	37	37	44	38	40	42
Liver and oesophageal tumour	0	0	0	0	0	1	5	16	13	17	21	21	20	20	19	32
Fatal liver tumour	2	1	0	5	1	4	8	15	7	17	24	24	22	29	23	32
Fatal oesophageal tumour	0	0	0	0	0	0	8	13	26	26	18	18	21	17	23	15
Fatal treatment-related tumour	2	1	0	5	1	4	16	28	33	43	42	42	43	46	46	47
Treatment-related tumour	11	4	0	9	2	9	26	38	38	47	45	45	48	47	47	47

Table 4.15 Incidence of treatment-related findings in female rats maintained on drinking water containing DEN

	No. of female rats with finding in group receiving (ppb DEN)															
	0	33	66	132	264	528	1060	1580	2110	2640	3170	4220	5280	6340	8450	16900
No. of rats	192	48	48	48	48	48	48	48	54	48	48	48	48	48	48	42
Liver tumour	17	7	4	3	8	13	32	39	44	42	38	41	34	40	45	39
Oesophageal tumour	0	0	0	0	0	3	17	20	28	23	34	30	31	37	33	26
Liver and oesophageal tumour	0	0	0	0	0	1	13	18	21	22	28	25	24	31	32	25
Fatal liver tumour	2	0	0	1	1	3	23	38	41	42	38	41	32	40	44	37
Fatal oesophageal tumour	0	0	0	0	0	1	3	3	8	1	6	5	9	5	2	3
Fatal treatment-related tumour	2	0	0	1	1	4	26	41	49	43	44	46	41	45	46	40
Treatment-related tumour	17	7	4	3	8	15	36	41	51	43	44	46	41	46	46	40

Figure 4.8 Each point represents the data from a group of 48 rats receiving the appropriate concentration of DEN in the drinking-water throughout their life.

Fig.4.8 RELATIONSHIP BETWEEN INCIDENCE OF LIVER TUMOURS AND DOSE OF DIETHYLNITROSAMINE

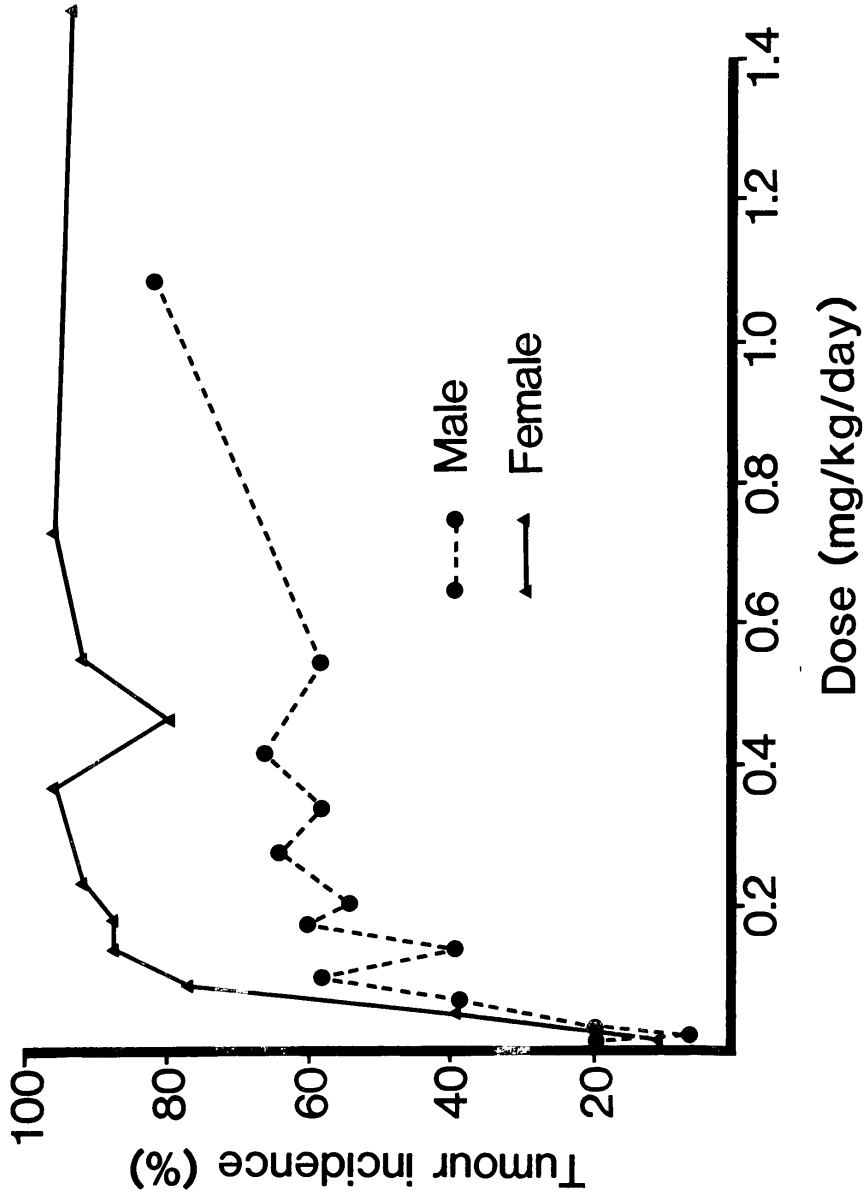


Figure 4.9 Each point represents the data from a group of 48 rats receiving the appropriate concentrations of DEN in the drinking water throughout their life.

Fig. 4.9 RELATIONSHIP BETWEEN INCIDENCE OF OESOPHAGEAL TUMOURS AND DOSE OF DIETHYLNITROSAMINE

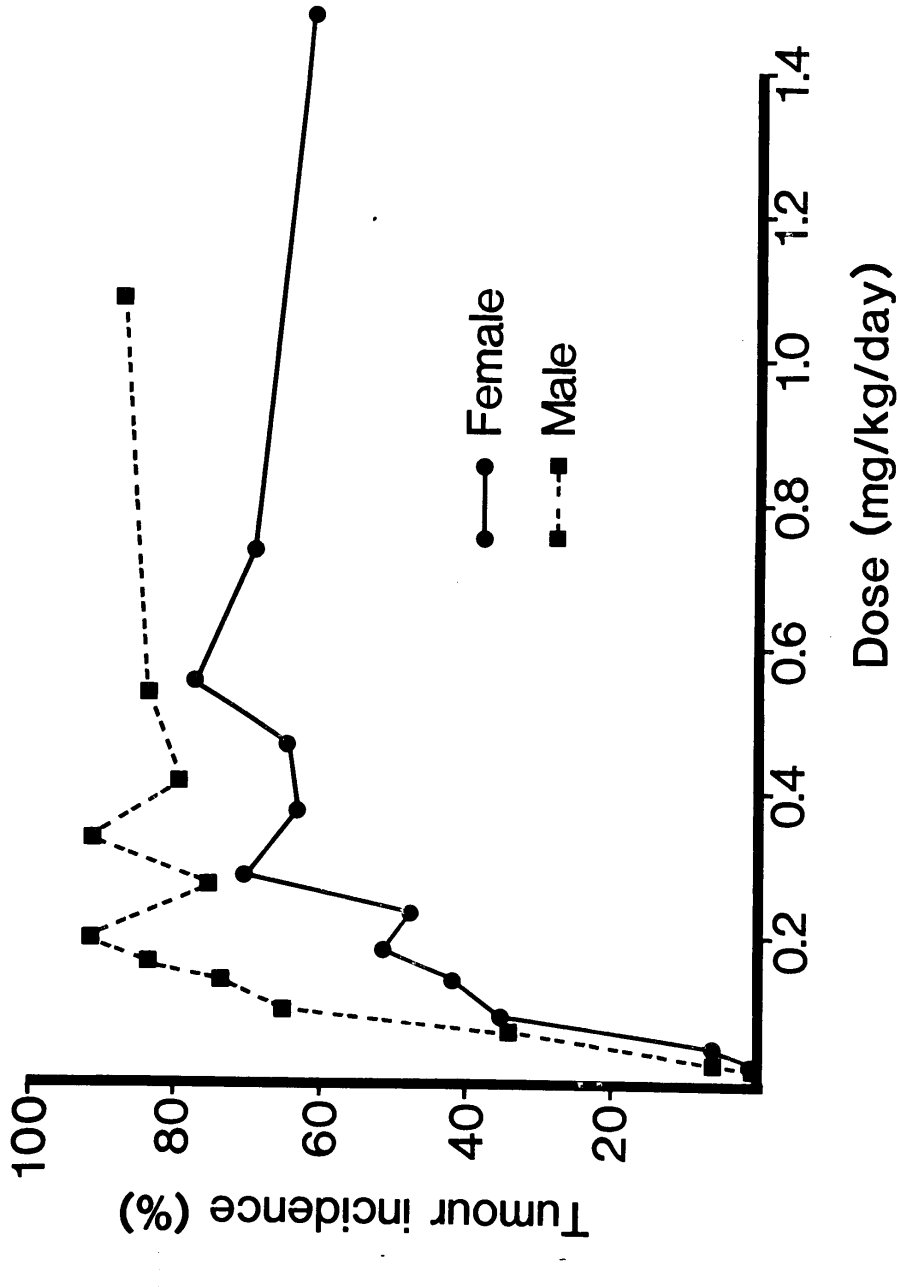
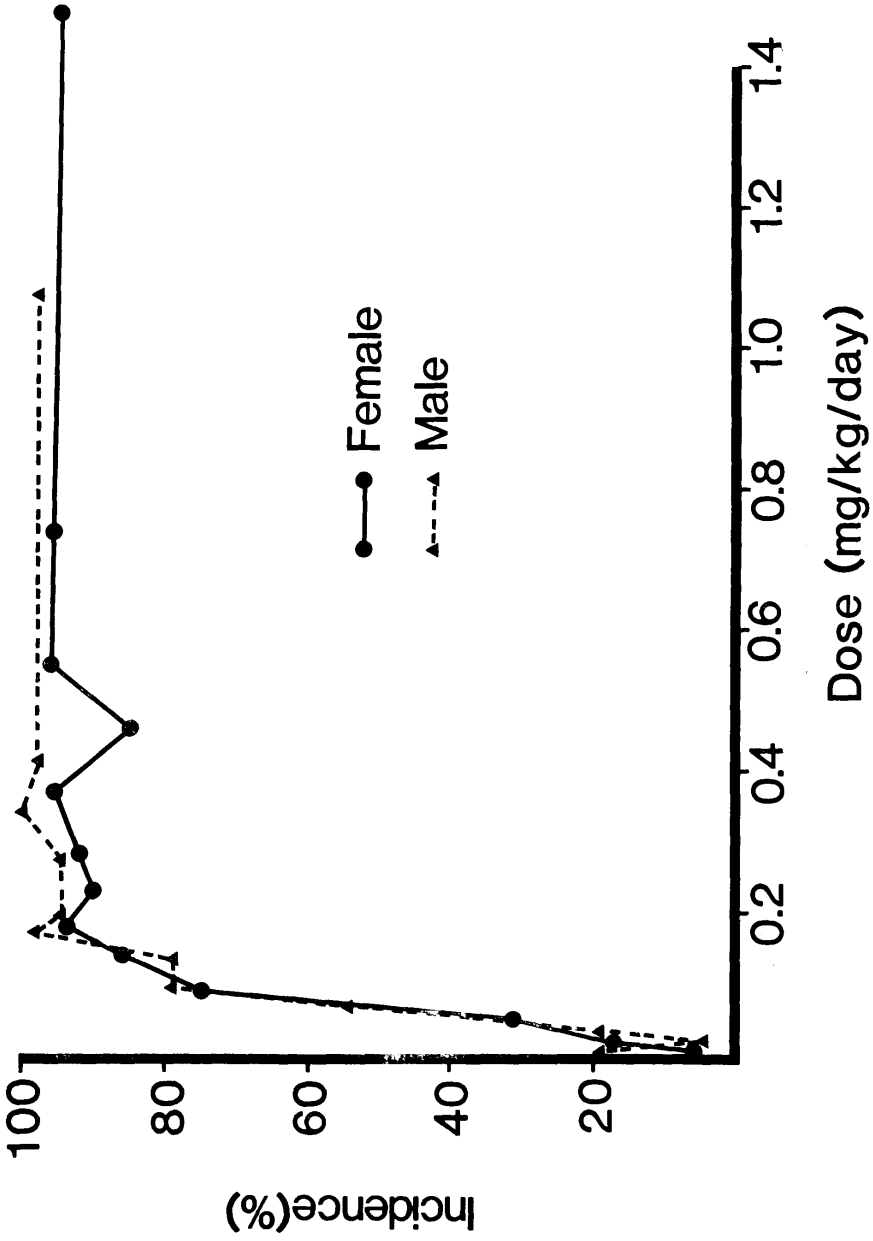


Figure 4.10 Each point represents the data from a group of 48 rats receiving the appropriate concentrations of DEN in the drinking-water throughout their life.

Fig. 4.10 RELATIONSHIP BETWEEN INCIDENCE OF ANIMALS WITH TREATMENT-RELATED TUMOURS AND DOSE OF DEN



of liver tumours in DMN-treated animals shows a similar picture (figure 4.11).

Relationship between dose and time of tumour

In view of the mortality data (table 4.2-4.5) an alternative analysis of this data might be based on time to tumour.

If such an analysis is to be successfully carried out the measure of time to tumour used must be unambiguous. A common approach to this problem, is to use the time at which the tumour causes death. This involves pathologists in the exercise of determining cause of death, an exercise criticised by many and relished by none. If for a moment we examine the reasons for requiring the cause of death information, it is to allow a distinction to be made between fully developed tumours and those at a much earlier stage of development. This distinction can be made without the need to define cause of death. Thus all tumours of similar size and development to those which were fatal are, for the purpose of analysis, regarded as fatal. Cumulative incidence plots have been drawn for the highest 4 doses of males given diethylnitrosamine (figure 4.12). This plot illustrates the similarity in shape between the curves obtained at different doses, together with the shift to the right with decreasing dosage. A detailed account of a method of analysis of this data, based on the cumulative incidence of tumours, is given in the next chapter.

Figure 4.11 Each point represents the data from a group of 48 rats receiving the appropriate concentration of DMN in the drinking-water throughout their life.

Fig. 4.11 RELATIONSHIP BETWEEN INCIDENCE OF LIVER TUMOURS AND DOSE OF DIMETHYLNITROSAMINE

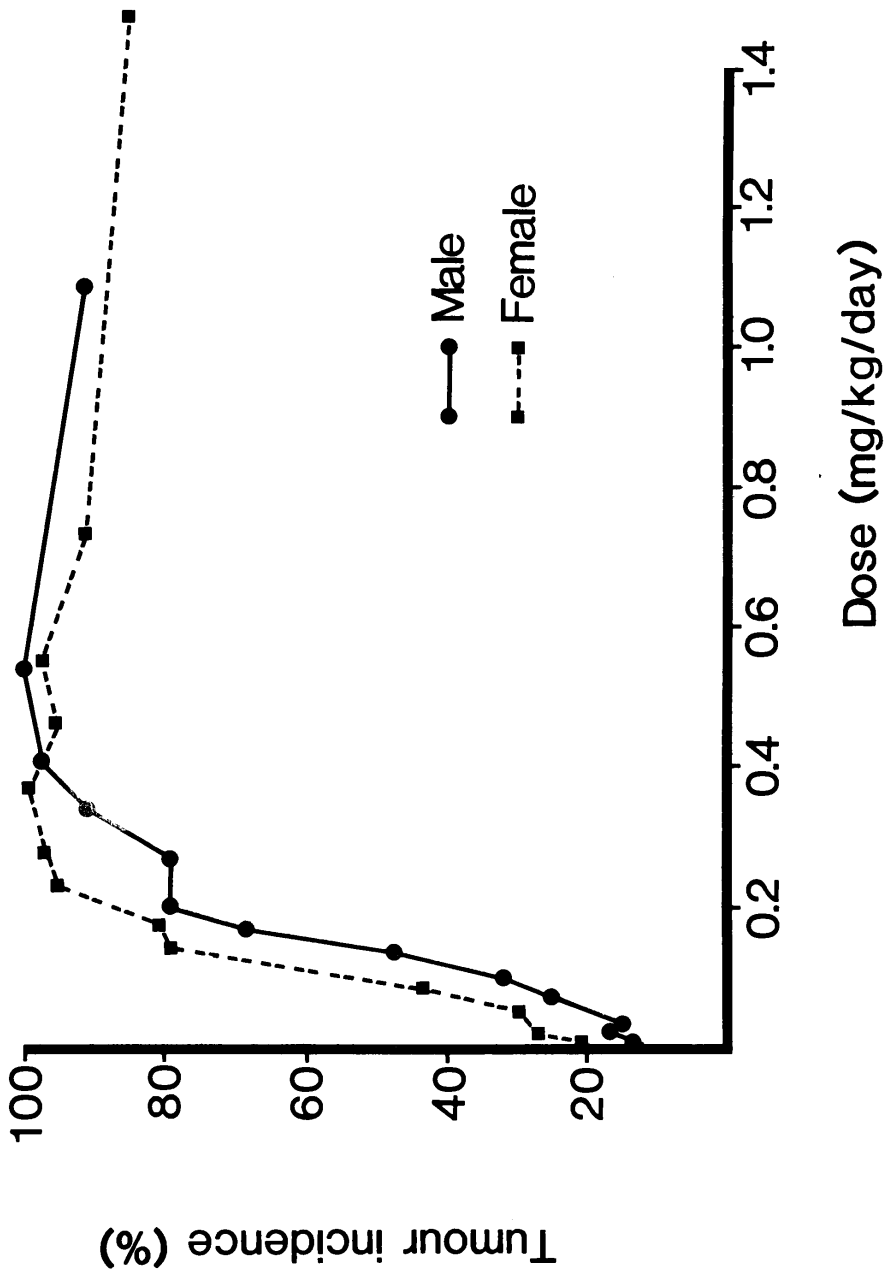
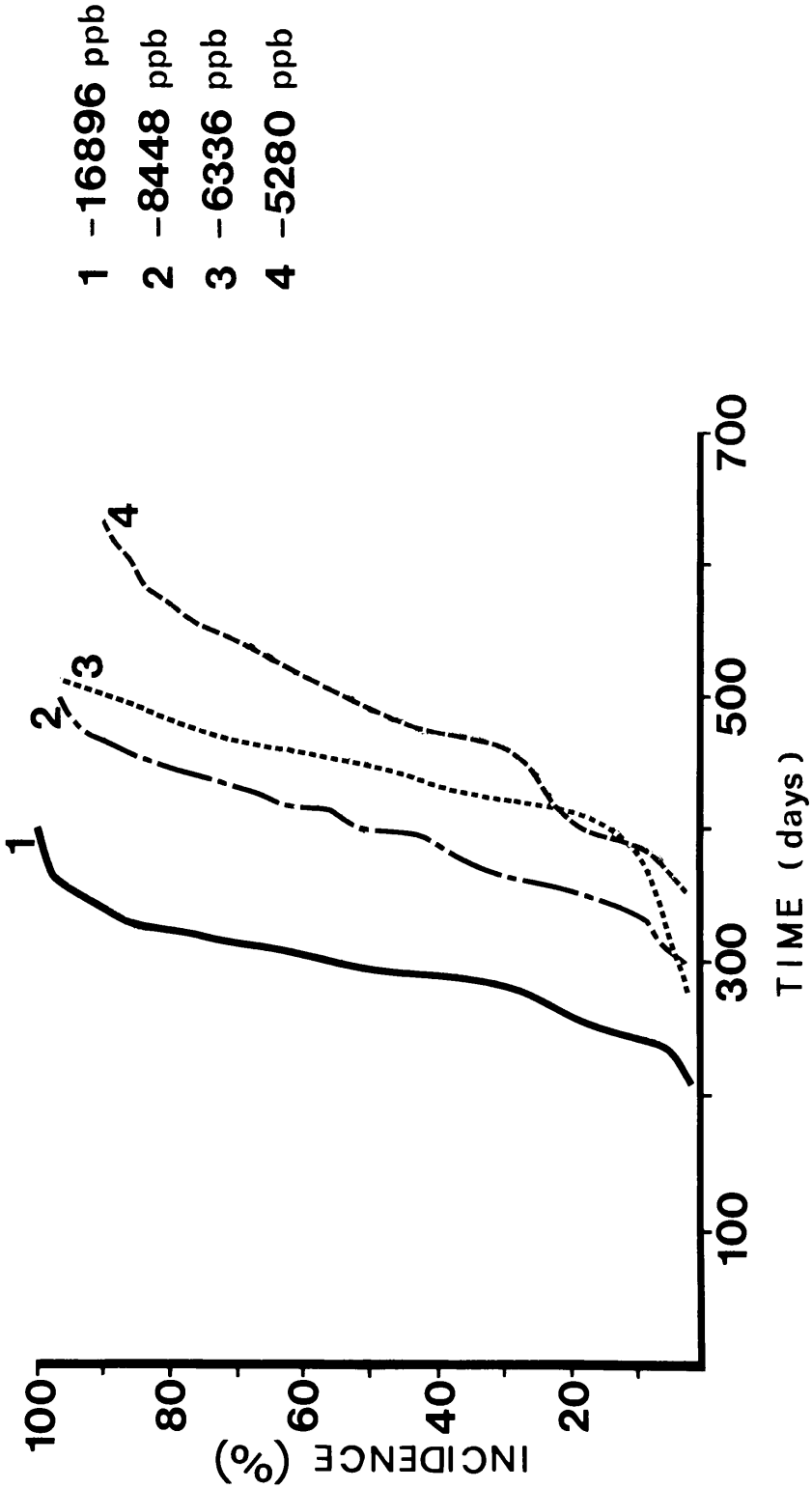


Fig. 4.12 CUMULATIVE INCIDENCE OF FATAL TREATMENT-RELATED TUMOURS IN MALE RATS RECEIVNG DEN IN THE DRINKING WATER



CHAPTER FIVE

ANALYSIS OF RESULTS

Objective of analysis

It is intended in this analysis to summarise the data from this study in such a way that it may be used to define the response that might be expected with any specified dose of nitrosamine.

Standardisation of cumulative incidence

For most of the high-dose groups the incidence of treatment-related tumours is close to 100%. Any deaths without tumours are usually very early in the study when no tumours would be expected. As tumours do not suddenly appear it is necessary to define a specific point in tumour development which will be comparable from one animal to another. The only practical point is the time at which the tumour causes the death of the animal. For all practical purposes, it was found that palpation was unlikely to detect tumours before the animal became ill. In all subsequent analysis this time will be referred to as 'the time of tumour'. As it is intended to compare the cumulative incidence curves at different doses a method has been developed to standardise these. The method used is based on that of Kaplan and Meier (1958) and permits the visualisation of the cumulative distribution as it would be if there were no competing causes of death. The method works in the following way:

On each day that a tumour occurs the following data is available;

(i) Number of animals alive at start of that day (N)

(ii) Number of deaths from a tumour during that day (X)

The tumour incidence rate for that day is then defined as:

$$\frac{X}{N} \times 100$$

On days when only deaths from other causes occur N is reduced but no incidence is calculated, e.g:

Day	Death from tumour	Death without tumour	No.of survivors	Tumour rate for day	Adjusted cumulative incidence
1		2	10	-	
2	1		8	$\frac{1}{8} \times 100 = 12.5\%$	12.5%
3	2		7	$\frac{2}{7} \times 100 = 28.6\%$	37.5%[a]
4		2	5	-	-
5	3		3	$\frac{3}{3} \times 100 = 100\%$	100.0%

[a]Value calculated as:

$$\left(\frac{[100 - 12.5] \times 28.6}{100}\right) + 12.5$$

The cumulative distributions adjusted in the above manner are shown for the highest doses of each sex of each nitrosamine in figures 5.1 - 5.4.

Figure 5.1 The cumulative incidence curves are coded 1 to 11 with each curve summarising the data from a single treatment group of 48 animals as below:

Code	Conc'n of DEN (ppm)	Code	Conc'n of DEN (ppm)
1	16.9	7	2.64
2	8.45	8	2.11
3	6.34	9	1.58
4	5.28	10	1.06
5	4.22	11	0.53
6	3.17		

Fig.5.1 ADJUSTED CUMULATIVE INCIDENCE OF FATAL TREATMENT-RELATED TUMOURS IN DEN-TREATED MALE RATS

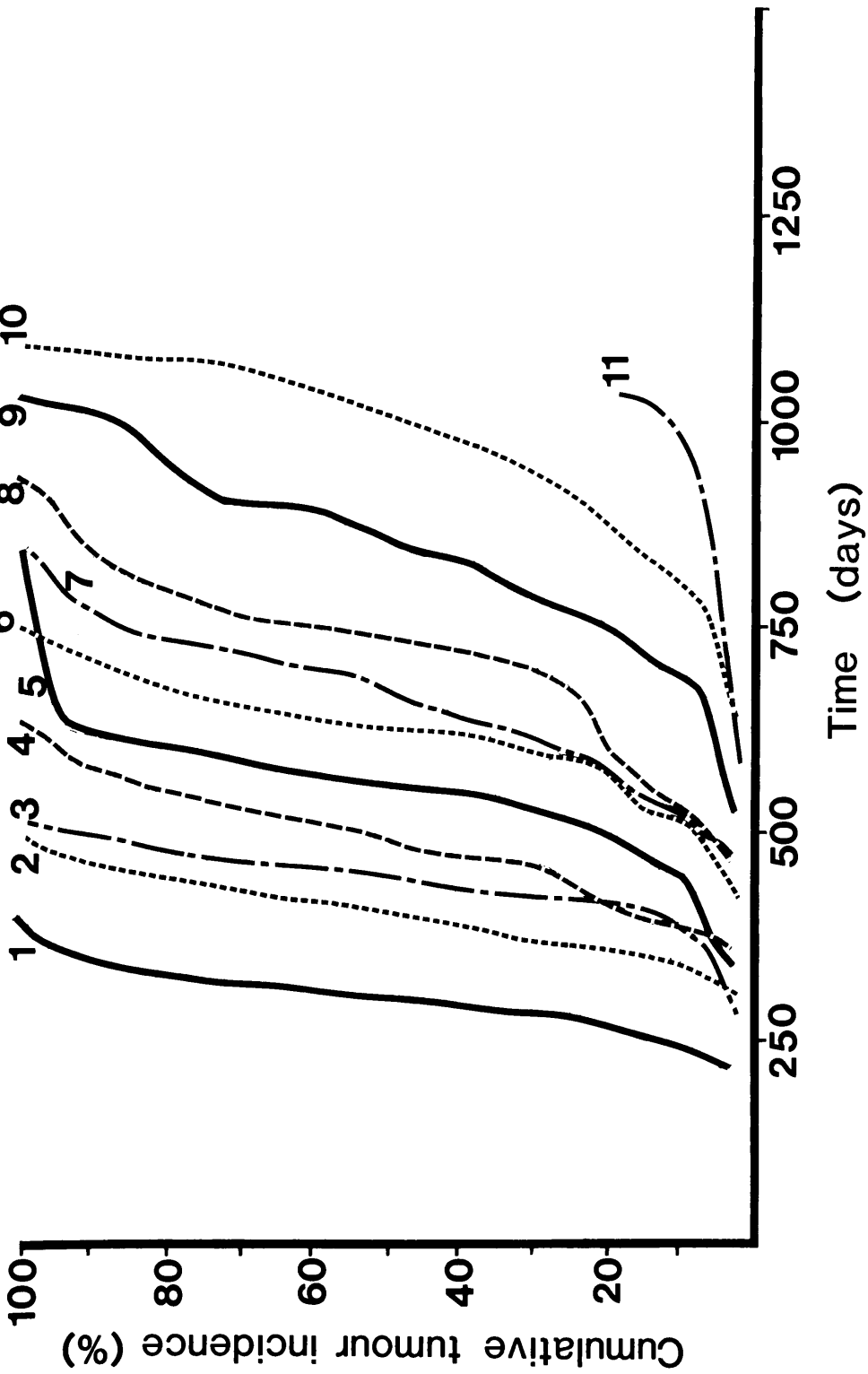


Figure 5.2 The cumulative incidence curves are coded 1 to 10 with each curve summarising the data from a single treatment group of 48 animals as below:

Code	Conc'n of DEN (ppm)	Code	Conc'n of DEN (ppm)
1	16.9	6	3.17
2	8.45	7	2.64
3	6.34	8	2.11
4	5.28	9	1.58
5	4.22	10	1.06

Fig. 5.2 ADJUSTED CUMULATIVE INCIDENCE OF FATAL TREATMENT-RELATED TUMOURS IN DEN-TREATED FEMALE RATS

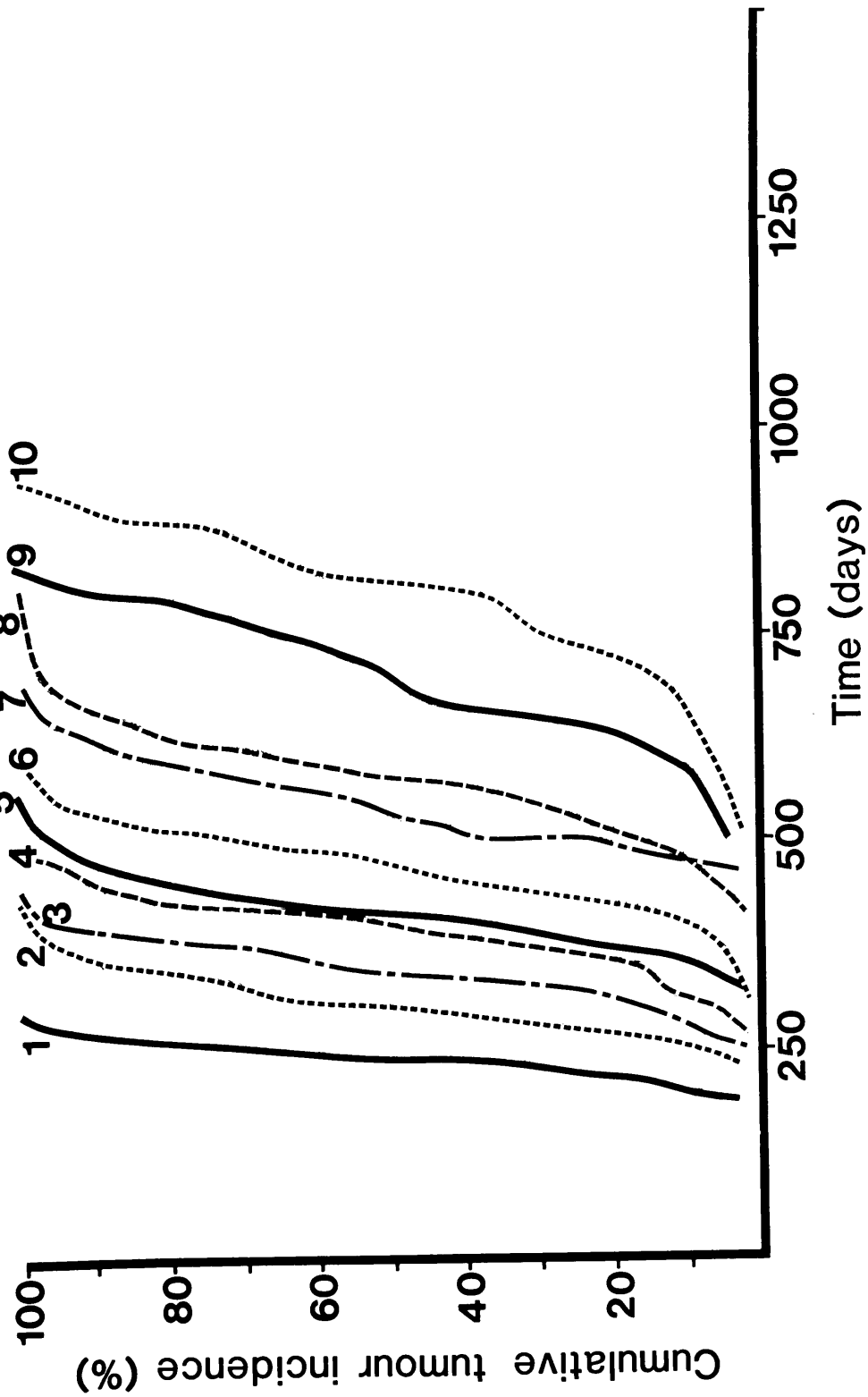


Figure 5.3 The cumulative incidence curves are coded 1 to 9 with each curve summarising the data from a single treatment group of 48 animals as below:

Code	Conc'n of DMN (ppm)	Code	Conc'n of DMN (ppm)
1	16.9	7	2.64
2	8.45	8	2.11
3	6.34	9	1.58
4	5.28		
5	4.22		
6	3.17		

Fig. 5.3 ADJUSTED CUMULATIVE INCIDENCE OF FATAL TREATMENT-RELATED TUMOURS IN DMN-TREATED MALE RATS

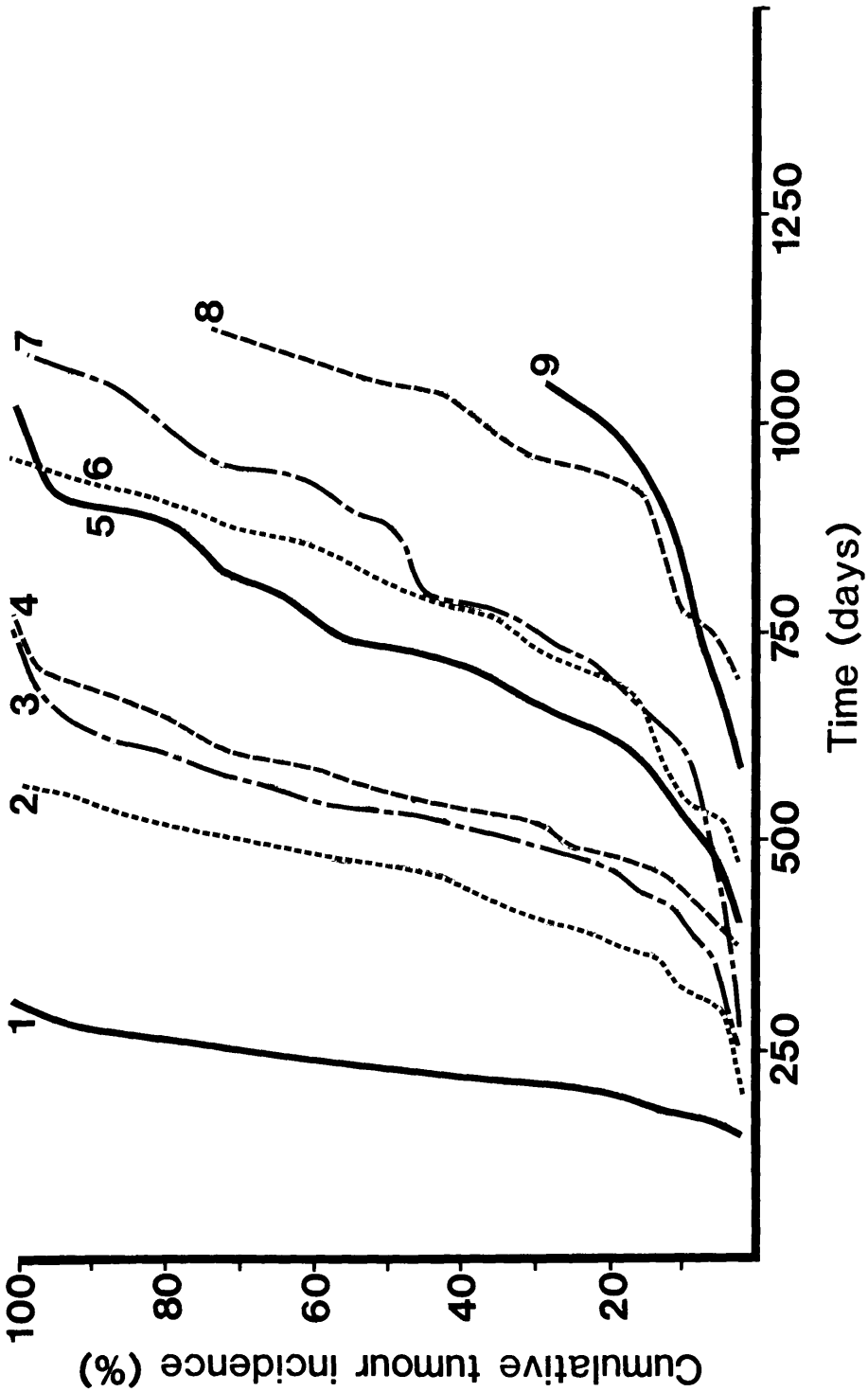
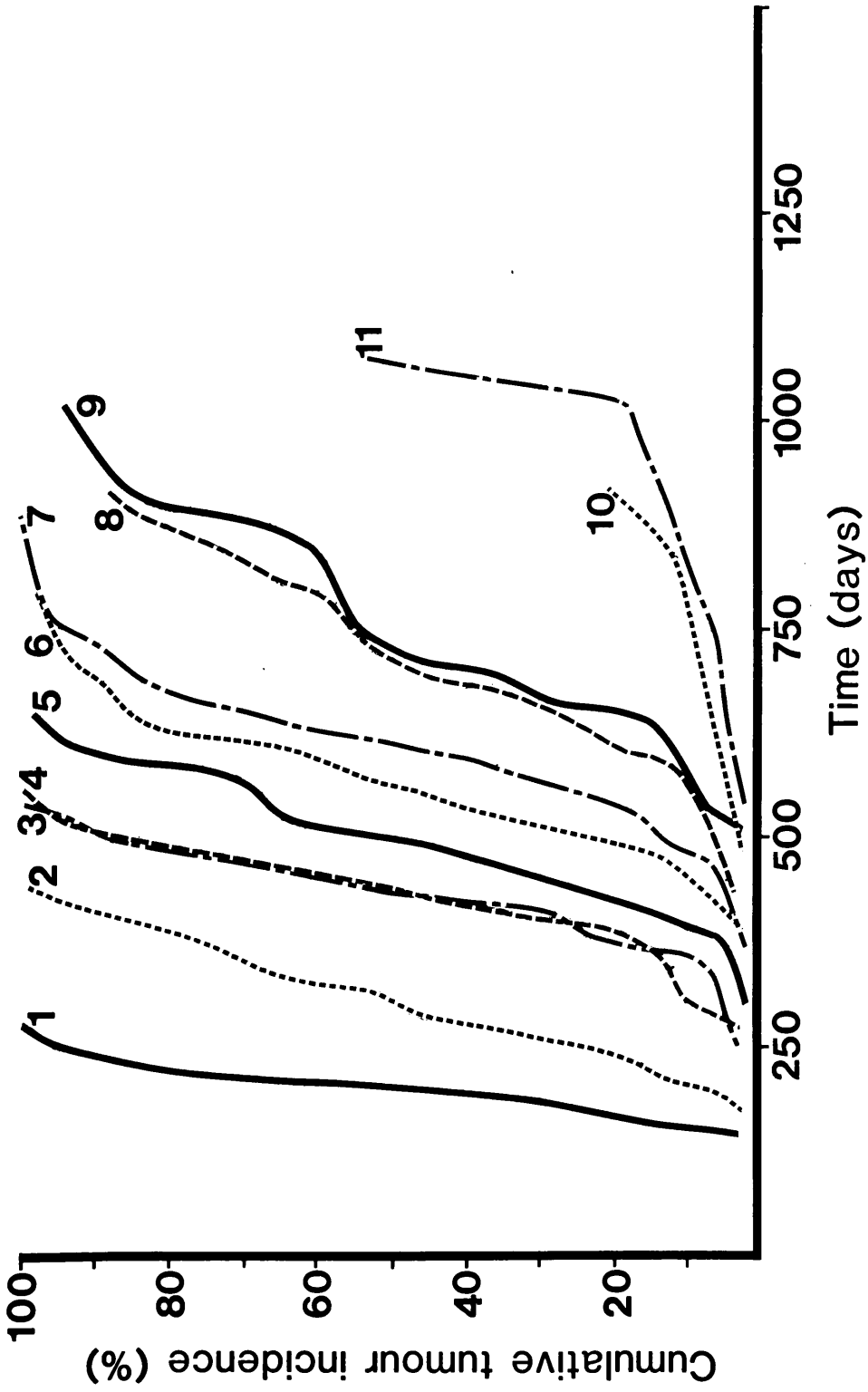


Figure 5.4 The cumulative incidence curves are coded 1 to 11 with each curve summarising the data from a single treatment group of 48 animals as below:

Code	Conc'n of DMN (ppm)	Code	Conc'n of DMN (ppm)
1	16.9	7	2.64
2	8.45	8	2.11
3	6.34	9	1.58
4	5.28	10	1.06
5	4.22	11	0.53
6	3.17		

Fig. 5.4 ADJUSTED CUMULATIVE INCIDENCE OF FATAL TREATMENT-RELATED TUMOURS IN DMN-TREATED FEMALE RATS



Median Time of Tumour

Part of the summary of the data from this study is to derive a relationship between the time of tumour and dose of nitrosamine. The most convenient summary of time of tumour is the median time for each group, which is the time at which 50% of the animals in that group are dead with a treatment-related tumour (t_{50}). Values for this time (t_{50}) were obtained directly from the cumulative plots for all high-dose groups. These values plotted against dose using $\log_e$ scales on both axes are shown in figures 5.5 and 5.6. A line was fitted to these plots by linear regression. From this linear regression a set of predicted median times was obtained for all groups. These are shown in tables 5.1 and 5.2, compared with the observed values. A correlation coefficient was calculated for the goodness of fit of the regression lines and was better than 0.99 in all cases.

Summarising the Cumulative Distribution

While the plot of median time against dose is useful in illustrating the existence of a relationship between dose and time of tumour, it is an incomplete summary of the data. In order to summarise the whole relationship it is necessary to incorporate some measure of the distribution of tumours about the median time.

A casual observation made upon the data provided the basis for the next stage of the analysis. It was noted that on a log/log plot of median times of tumour against dose, if time to 10% or 90% tumours was plotted these gave lines parallel to that given by median time but set to the left and

Table 5.1 Predicted values for median time of tumour in male rats obtained from the linear regression of actual median time on dose of nitrosamine

	Median time (days) at dose of nitrosamine (mg/kg/day)														
	1.08	0.54	0.41	0.34	0.27	0.20	0.17	0.14	0.10	0.07	0.03	0.017	0.008	0.004	0.002
DEN - predicted	285	393	446	486	540	620	668	730	852	1004	1483	1925	2722	3743	5148
DEN - observed	288	386	444	488	549	618	662	729	851	-	-	-	-	-	-
DMN - predicted	251	411	500	571	672	831	933	1070	1359	1750	3190	4772	8144	13312	21760
DMN - observed	224	469	529	550	729	803	879	1045	-	-	-	-	-	-	-

observed median time - is the time at which the adjusted cumulative incidence reaches 50%

predicted median time - is derived from the linear regression of $\log_e$ observed median time on $\log_e$ mg/kg/day dose.

The correlation coefficient for the fit of the regression line to the observed data was > 0.99 in both cases.

Table 5.2 Predicted values for median time of tumour in female rats obtained from the linear regression of observed median time on dose of nitrosamine

	Median time (days) at dose of nitrosamine (mg/kg/day)														
	1.47	0.73	0.55	0.46	0.37	0.28	0.23	0.18	0.14	0.09	0.05	0.02	0.011	0.006	0.003
DEN - predicted	228	313	355	385	425	483	528	590	661	807	1054	1597	2094	2756	3773
- observed	239	301	344	403	412	467	529	572	709	-	-	-	-	-	-
DMN - predicted	218	322	377	417	470	550	614	704	810	1037	1440	2404	3358	4712	6942
- observed	199	308	435	427	494	556	611	713	730	-	-	-	-	-	-

observed median time - is the time at which the adjusted cumulative incidence reaches 50%

predicted median time - is derived from the linear regression of $\log_e$ observed median time on $\log_e$ mg/kg/day dose.

The correlation coefficient for the fit of the regression line to the observed data was > 0.99 in both cases.

Fig.5.5 RELATIONSHIP BETWEEN DOSE OF DIETHYLNITROSAMINE AND TIME OF TUMOUR

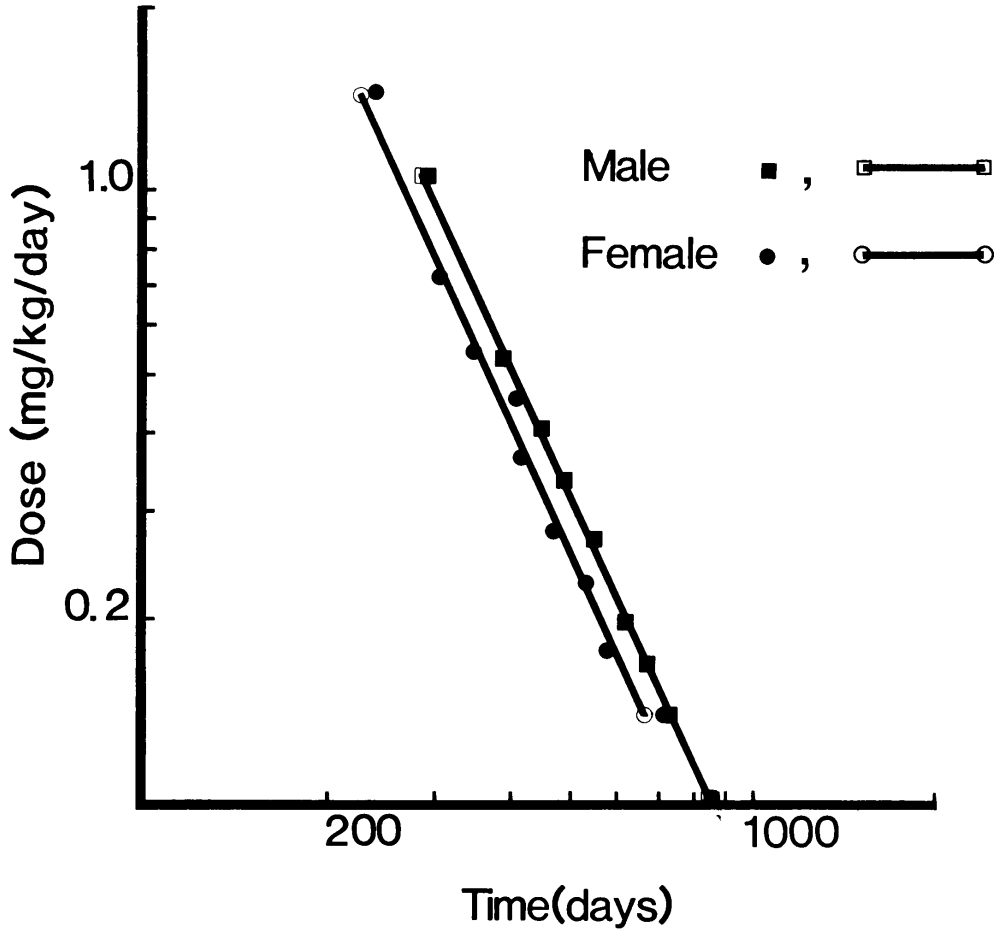
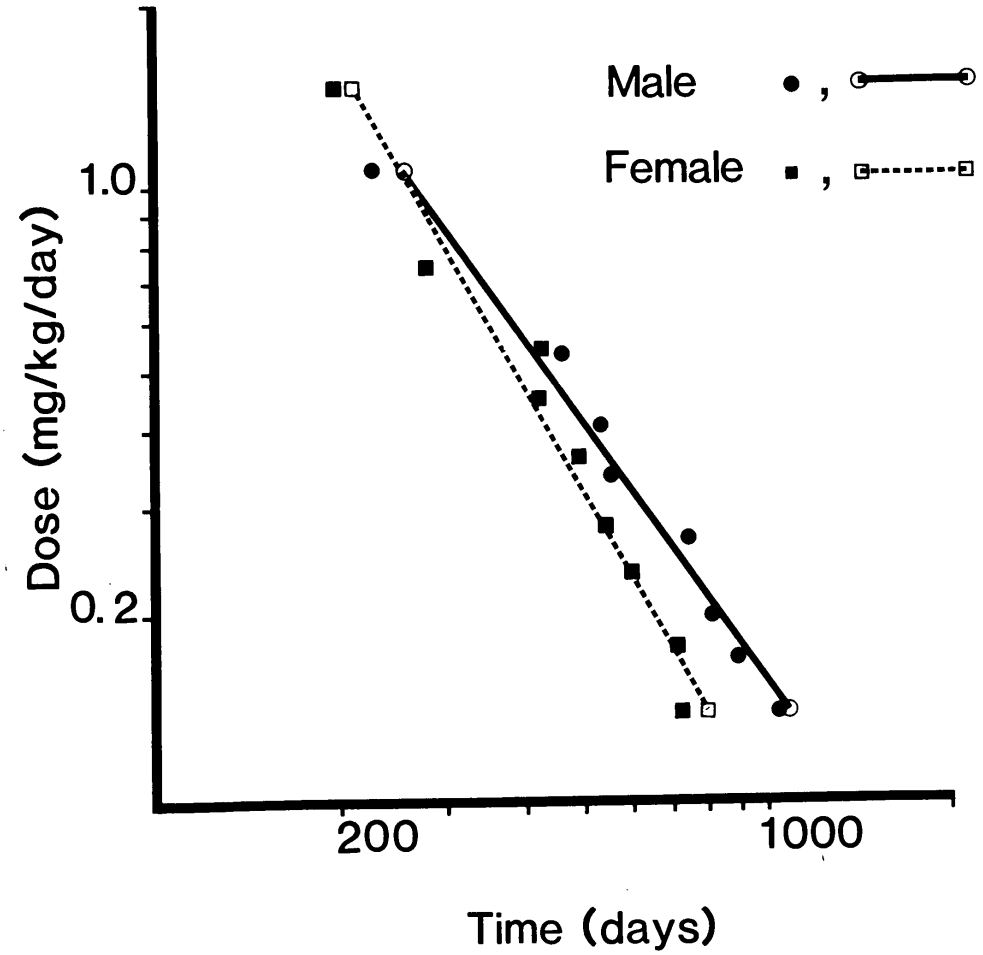


Fig.5.6 RELATIONSHIP BETWEEN DOSE OF DIMETHYLNITROSAMINE AND TIME OF TUMOUR



right respectively.

It was discovered that this is equivalent to the observation that the time taken to reach x% tumours is a constant proportion of the time taken to reach 50% tumours. This feature can therefore be used to describe the cumulative distribution for any dose of the particular nitrosamine. For each sex and each of the highest 6 treatment groups, the times that tumour incidence reached the following cumulative incidences were read from the graphs:

2% ; 10% ; 20%

30% ; 40% ;

60% ; 70% ; 80%

90% ; 100%.

The ratio between each of those times and t_{50} was calculated. For each sex of each nitrosamine all the ratios for each incidence point were meaned. Comparison of the results for both sexes on each nitrosamine should produce similar curves, thus the data from both sexes were pooled to give a single curve for each nitrosamine. These are shown in figures 5.7 and 5.8 and are subsequently referred to as the Standard Curves for each nitrosamine.

Use of the Summary

The combination of the relationship between dose and time of tumour and the standard curves allows a complete summary of the distribution of tumours at any dose of either DMN or DEN. The predicted distribution of tumours in time for any dose is derived by the following process:

(i) obtain the value for the t_{50} from the line

Figure 5.7

The curve is derived from the ratio of median tumour time (t_{50}) to time at which specified cumulative incidences are reached (for data see table 5.3). Each point is the mean of values from 18 treatment groups.

Fig.5.7 Standard curve for the cumulative incidence of treatment-related tumours in DEN-treated rats.

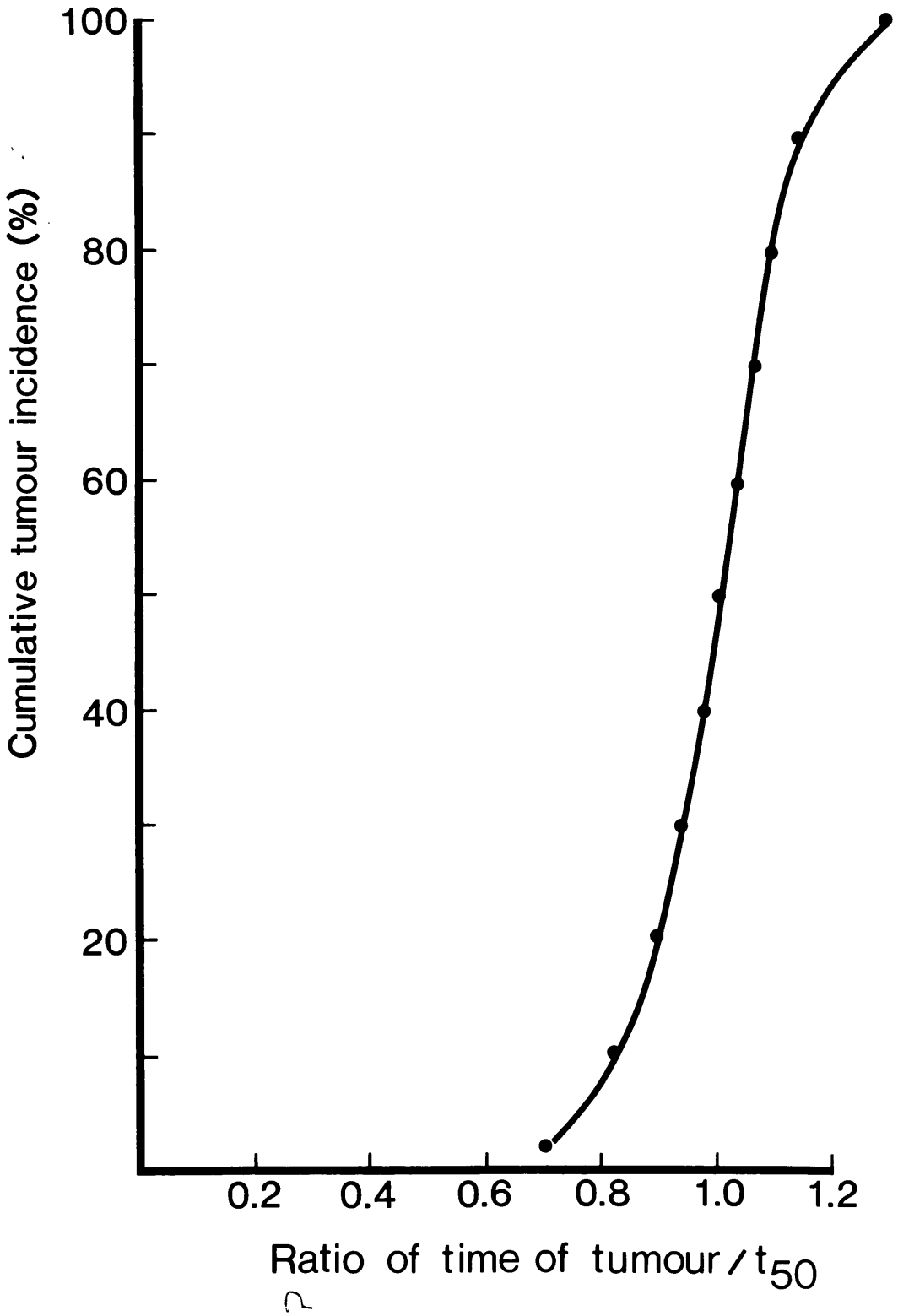
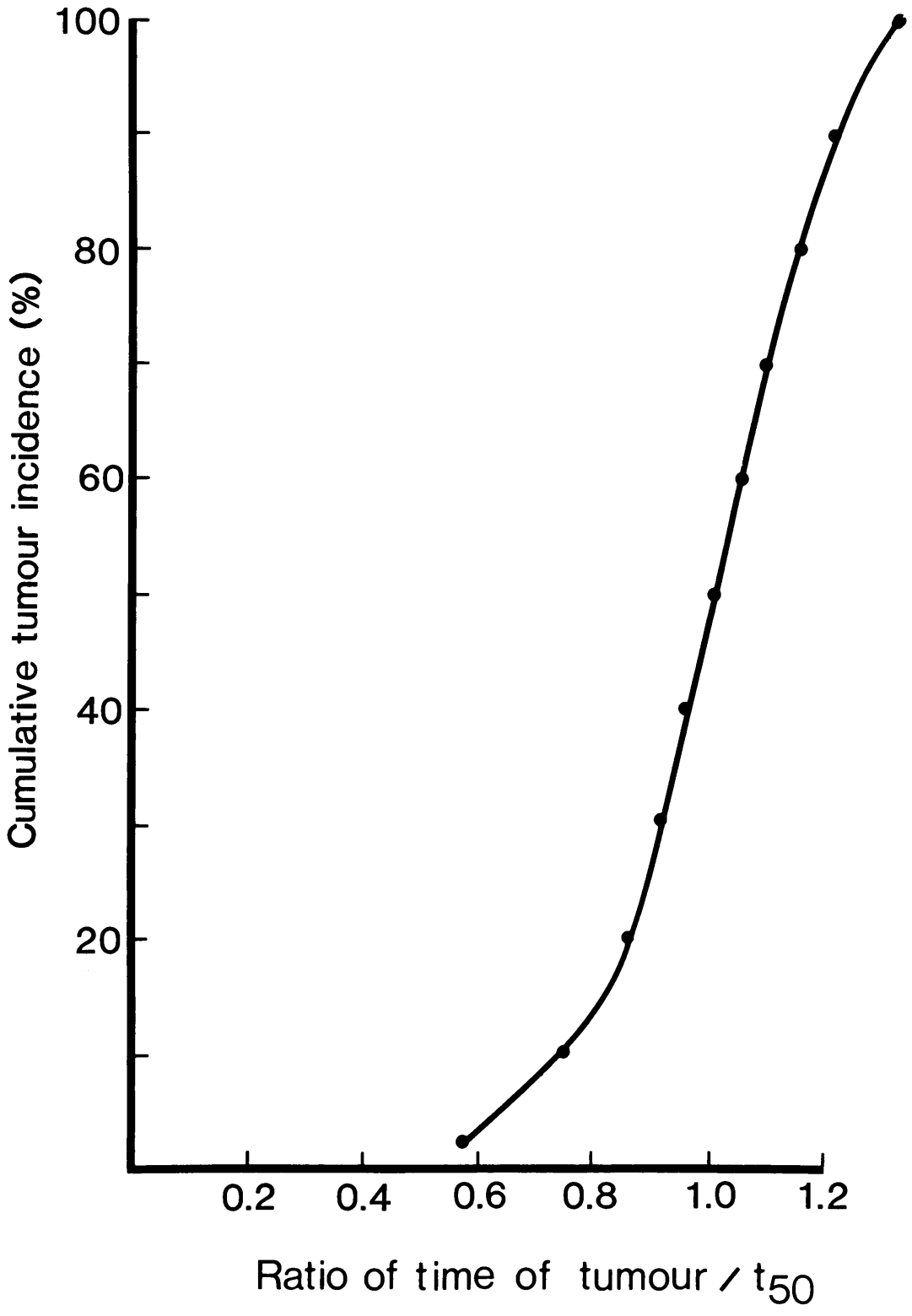


Figure 5.8

The curve is derived from the ratio of median tumour time (t_{50}) to time at which specified cumulative incidences are reached (for data see table 5.3).
Each point is the mean of values from 16 treatment groups.

Fig.5.8 Standard curve for the cumulative incidence of treatment-related tumours in DMN-treated rats.



relating dose to median tumour time.

(ii) Using the proportions of the standard curve as set out in table 5.3 below, the times that cumulative tumour incidence reaches 2, 10, 20, 30, 40, 60, 70, 80 and 90% can be obtained.

Table 5.3 Ratio between median tumour time (t_{50}) and time at which specified cumulative tumour incidences are reached

	Ratio between t_{50} and time cumulative tumour incidence reaches:									
	2%	10%	20%	30%	40%	50%	60%	70%	80%	90%
DEN	0.697	0.816	0.891	0.935	0.968	1	1.029	1.059	1.098	1.137
DMN	0.570	0.748	0.850	0.910	0.951	1	1.054	1.102	1.154	1.215

Application of the standard curves for each nitrosamine to the calculated t_{50} values produces a set of curves as shown in figure 5.9. These demonstrate a similar pattern to that seen in the original data but are in fact of little value in predicting events at low doses, where tumour incidence is certainly considerably less than 100%. The simplest reason for tumour incidence to be lower than 100% is that a proportion of the animals die before the time that tumours appear. To explore the contribution of natural mortality, a technique was developed to correct the predicted cumulative tumour incidence curve at any dose, for this additional influence.

Correction of Predicted Curves for Natural Mortality

The estimate of expected natural mortality is based on the cumulative mortality data from the relevant control group.

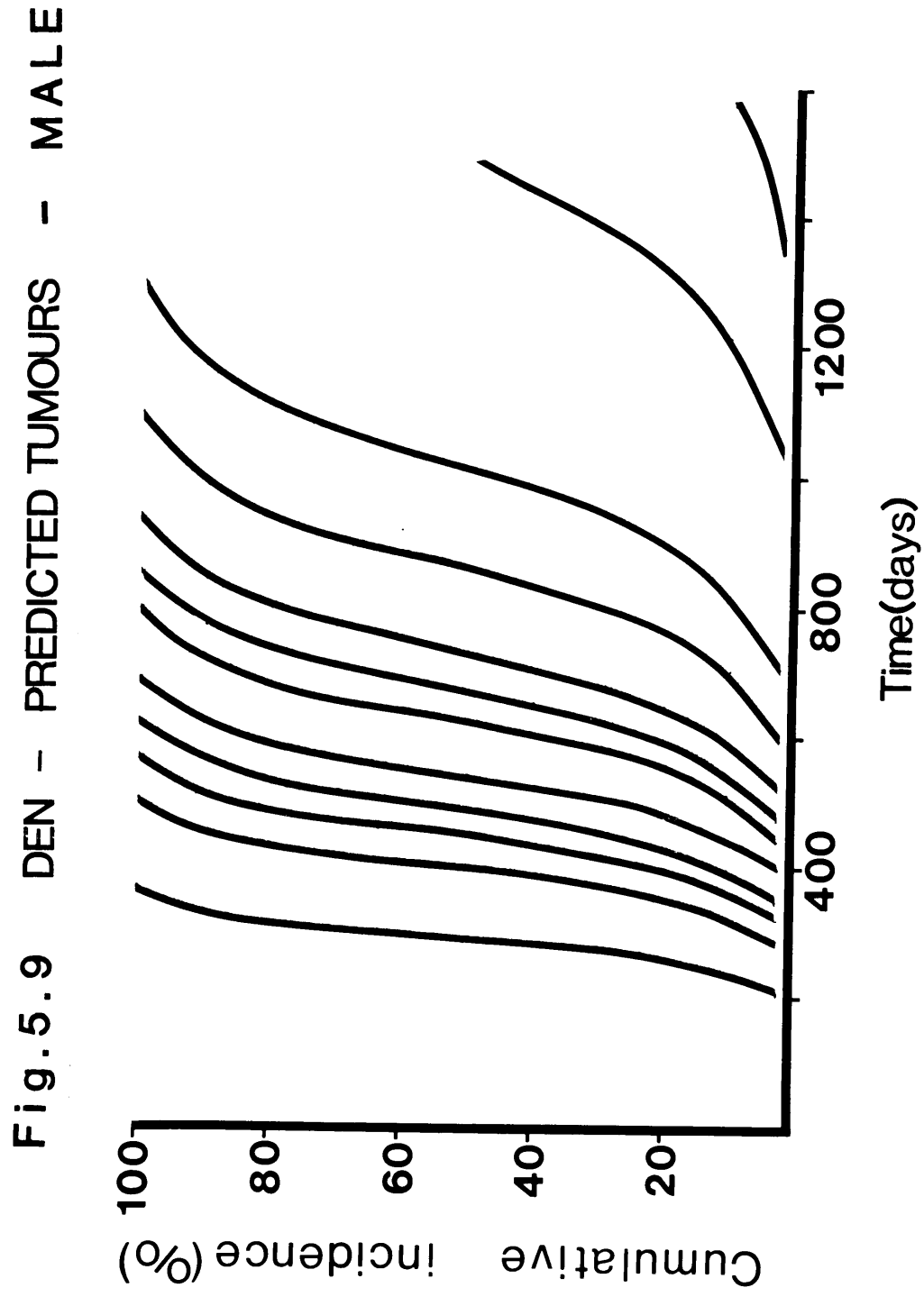


Fig.5.9 DEN - PREDICTED TUMOURS - MALE

The lifespan was divided into a series of 50-day intervals starting at day 0, the first day of treatment, and ending at day 1400 when the last rat had died. For each interval the expected death rate within that interval was derived from the cumulative mortality data as below:-

M_S = % Cumulative mortality at start of interval

M_E = % Cumulative mortality at end of interval

S_S = % Survival at start of interval [$S_S = 100 - M_S$]

DR = Death rate [%] within interval

$$DR = \frac{M_E - M_S}{S_S} \times 100$$

Thus DR% of animals alive at the start of the interval will be dead at the end of the interval.

By a similar calculation, using values obtained from the predicted cumulative tumour incidence curve, the expected tumour rate within each interval was derived:

T_S = % Cumulative tumour incidence at start of interval

T_E = % Cumulative tumour incidence at end of interval

S_S = % Surviving tumour-free at start of interval

TR = Tumour rate [%] within interval.

$$TR = \frac{T_E - T_S}{S_S} \times 100$$

Thus TR% of animals surviving tumour-free at the start of the interval will have died from a tumour by the end of the interval.

The two rates DR and TR are assumed to act totally independently within each interval and are regarded as competing for the death of the animals. The following example in table 5.4 demonstrates how these rates can be applied to a hypothetical animal population to give expected values for the number of animals dying without tumours, and the number dying from tumours.

The figure given for number of survivors at the start of each interval (N) is calculated by subtracting the predicted numbers of deaths, both from tumours and other causes, in the previous interval from the number alive at the start of that interval. An allowance is made for the fact that regarding the two rates DR and TR as operating independently will lead to some animals being predicted as killed twice (i.e. from tumour and other causes in the same interval). Thus for interval i where preceding interval is i - 1:

$$N_i = N_{i-1} - A_{i-1} - B_{i-1} + \left(\frac{TR_{i-1} \times DR_{i-1} \times N_{i-1}}{10000} \right)$$

where $A_i = \frac{DR_i \times N_i}{100}$

and $B_i = \frac{TR_i \times N_i}{100}$

Calculations similar to the one shown above were performed for the predicted curves for all doses used on the study, where the first tumour was predicted to occur within the normal maximum lifespan (1400 days). The resultant calculated incidences are compared with observed values in figures

Table 5.4 An example of calculation of predicted numbers of tumours

	DR	TR	N	A	B
Interval	Death-rate (%)	Tumour-rate (%)	No. of survivors @ start of interval	No. of dead	No. of tumours
1	1	2	48	0.48	0.96
2	3	10	46.57	1.397	4.657
3	5	20	40.66	2.033	8.132
4	9	30	30.80	2.772	9.24
5	15	60	19.62	2.943	11.772
			Totals	9.625	34.761

DR = Death-rate (%) within interval derived from control mortality data.

TR = Predicted tumour-rate (%) within interval derived from predicted cumulative tumour incidence curve.

N = Number of animals alive at start of interval derived as the number alive at start of preceding interval less the total predicted deaths within that interval

A = Predicted number of animals dying from causes other than an induced tumour, during the interval.

B = Predicted number of animals dying from an induced tumour during the interval.

5.10 - 5.13. In all cases the predicted curves fit the observed data sufficiently closely for the lack of tumours at low doses to be considered totally explicable by the failure of animals to survive in sufficient numbers to a time when those doses would induce a detectable incidence of tumours.

While various alternative explanations could be offered to explain the decline in incidence at low doses the results of this analysis confirm that the data from this study was not incompatible with the following assumptions:

(i) That all doses of both DEN and DMN used in this study have the potential to induce tumours in 100% of exposed animals if those animals survive long enough for the tumours to appear.

(ii) The failure of animals to survive long enough is sufficient explanation for all incidences of tumours less than 100%

(iii) The major response factor in any dose-response relationship with these nitrosamines is time.

Figure 5.10

The solid black line and solid points are the predicted curve and values for tumour incidence. Observed data is represented by open circles.

Fig. 5.10 COMPARISON OF OBSERVED AND EXPECTED INCIDENCES OF FATAL TREATMENT-RELATED TUMOURS IN DEN-TREATED MALE RATS

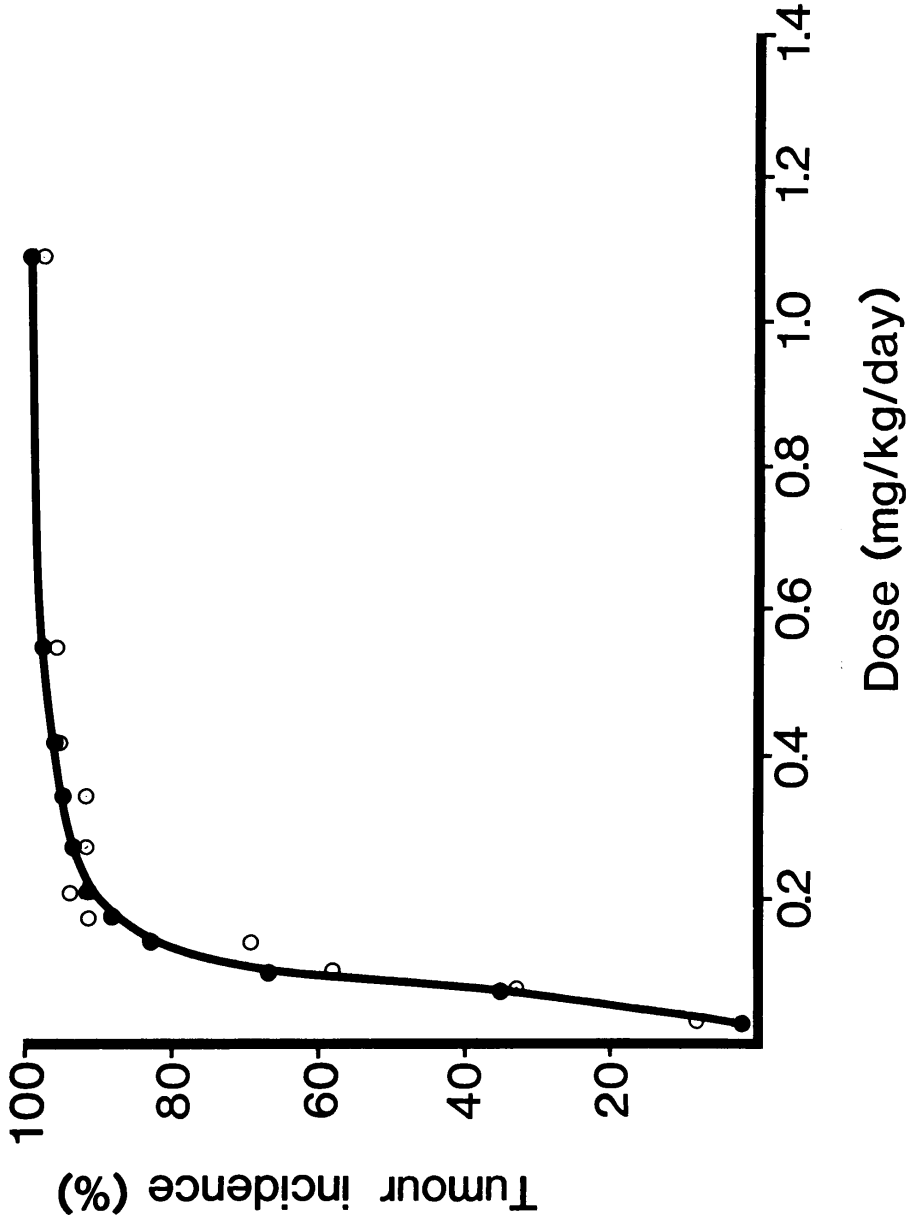


Figure 5.11

The solid black line and solid points are the predicted curve and points for tumour incidence.

The open circles are the observed data.

Fig. 5.11 COMPARISON OF OBSERVED AND EXPECTED INCIDENCES OF FATAL TREATMENT-RELATED TUMOURS IN DEN-TREATED FEMALE RATS

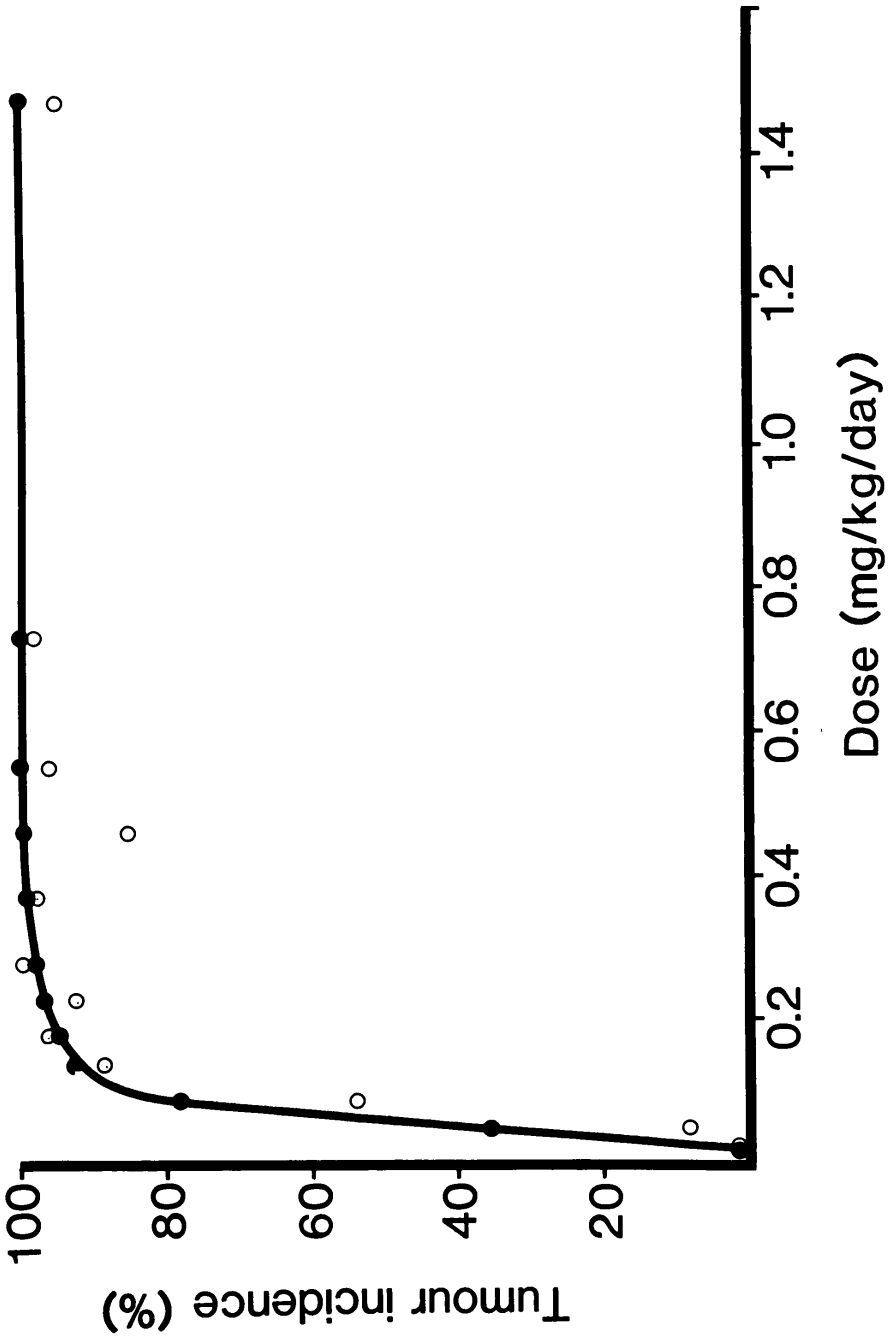


Figure 5.12

The solid black line is the predicted curve of tumour incidence against dose.

The open circles are the observed data.

Fig. 5.12 COMPARISON OF OBSERVED AND EXPECTED INCIDENCES OF FATAL TREATMENT-RELATED TUMOURS IN DMN-TREATED MALE RATS

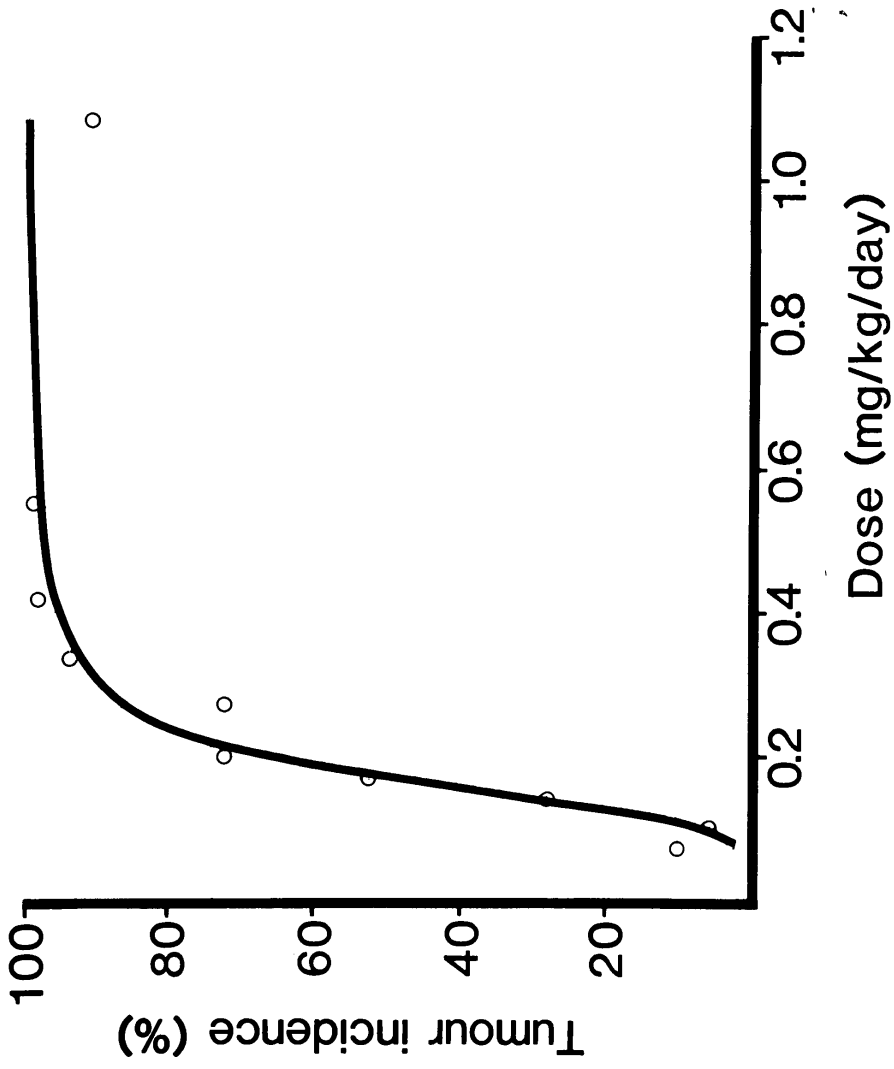
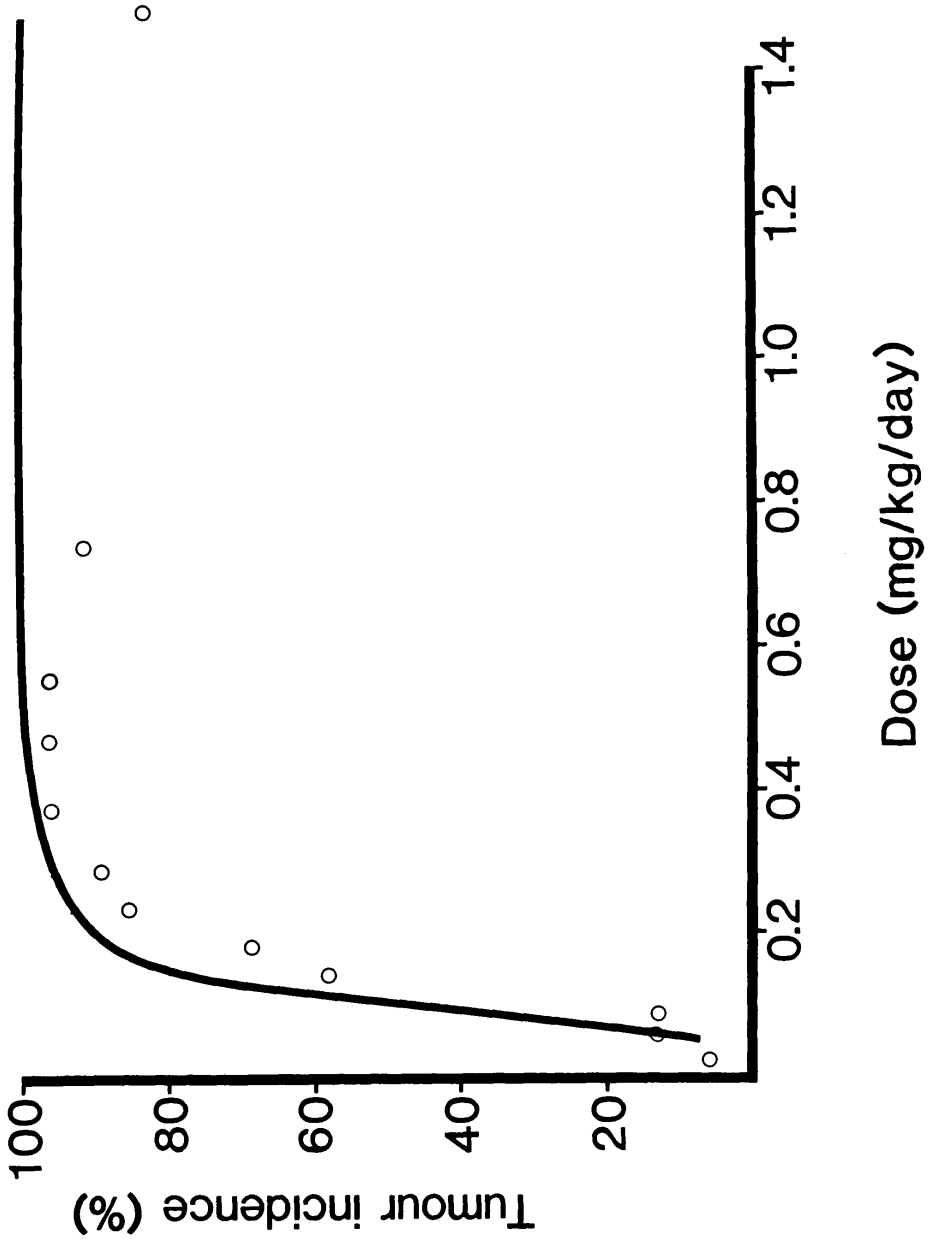


Figure 5.13

The solid black line is the predicted curve of tumour incidence against dose.

The observed data is shown by open circles.

Fig 5.13 COMPARISON OF OBSERVED AND EXPECTED INCIDENCES OF FATAL TREATMENT-RELATED TUMOURS IN DMN-TREATED FEMALE RATS



CHAPTER SIX

DISCUSSION

Tumour Types and Incidences

In general, the type and site of the tumours induced in this study was similar to that described for DEN by Druckrey [1967] and for DMN by Barnes and Magee [1956]. For the first time, however, this study demonstrated the potential of these carcinogens to cause tumours of every cell type present in the liver. For all cell types, apart from the biliary epithelium both malignant and benign tumours were seen. In the case of the biliary epithelium, while both nitrosamines induced numerous benign cystadenomas very few malignant tumours were seen.

The pattern of the biliary tumours, particularly in the mid-dose DMN groups, where they were the commonest lesion, may be a confusing factor in the analysis of the dose-response pattern in these groups. While all other fatal, induced tumours show similar time distributions at similar doses the biliary cystadenomas appear to become palpable and thus fatal earlier at each dose than tumours of other cell types. It is likely, therefore, that the dose-response relationships derived from this data would overestimate the incidence of liver tumours at doses below those at which the biliary tumours are common.

With DEN the tumours of the oesophagus showed a clear cut-off point below which dose no lesion of the oesophageal epithelium was seen. The main reason for the clarity of this point is the total absence of spontaneous tumours of this epithelium in untreated rats. While such a clear cut-off indicates some form of effect threshold for this tissue

this may have many origins; and cannot be considered relevant to discussions of true thresholds of effect when other organs in the same animals were affected by treatment. It is possible that below a dose of 500 ppb the liver succeeds in metabolising so much of the ingested nitrosamine that the effective dose to the oesophagus is reduced to a value close to zero. It is also possible that a proportionate dose of nitrosamine does reach the oesophagus but is too small to cause sufficient damage for the repair processes to be overwhelmed. It is likely that a prime requirement for oesophageal tumours is a dose high enough to bring about epithelial hyperplasia and ulceration (Napalkov and Pozharisski, 1969) and that the tumour development does not proceed in the absence of stimulus until a relatively late stage. This would separate the tumour development process in the oesophagus from that in other tissues such as the liver. With nitrosamines there is little evidence for the need for hepatocellular hyperplasia as a precursor of malignant tumours, although this is known to be the mechanism of hepatocarcinogenesis with some other chemicals (e.g. Ponceau MX). It is therefore concluded that the cut-off point for oesophageal tumours does not indicate the existence of an overall threshold of response to DEN. The details of tumour response in the liver show a background of spontaneous tumour incidence in untreated animals, making the detection of the very few additional tumours, predicted to occur at the lowest doses, impossible.

At most doses of DEN the tumours of the two main target sites appeared to compete for the death of the animal, thus at these doses a large proportion of the treated animals

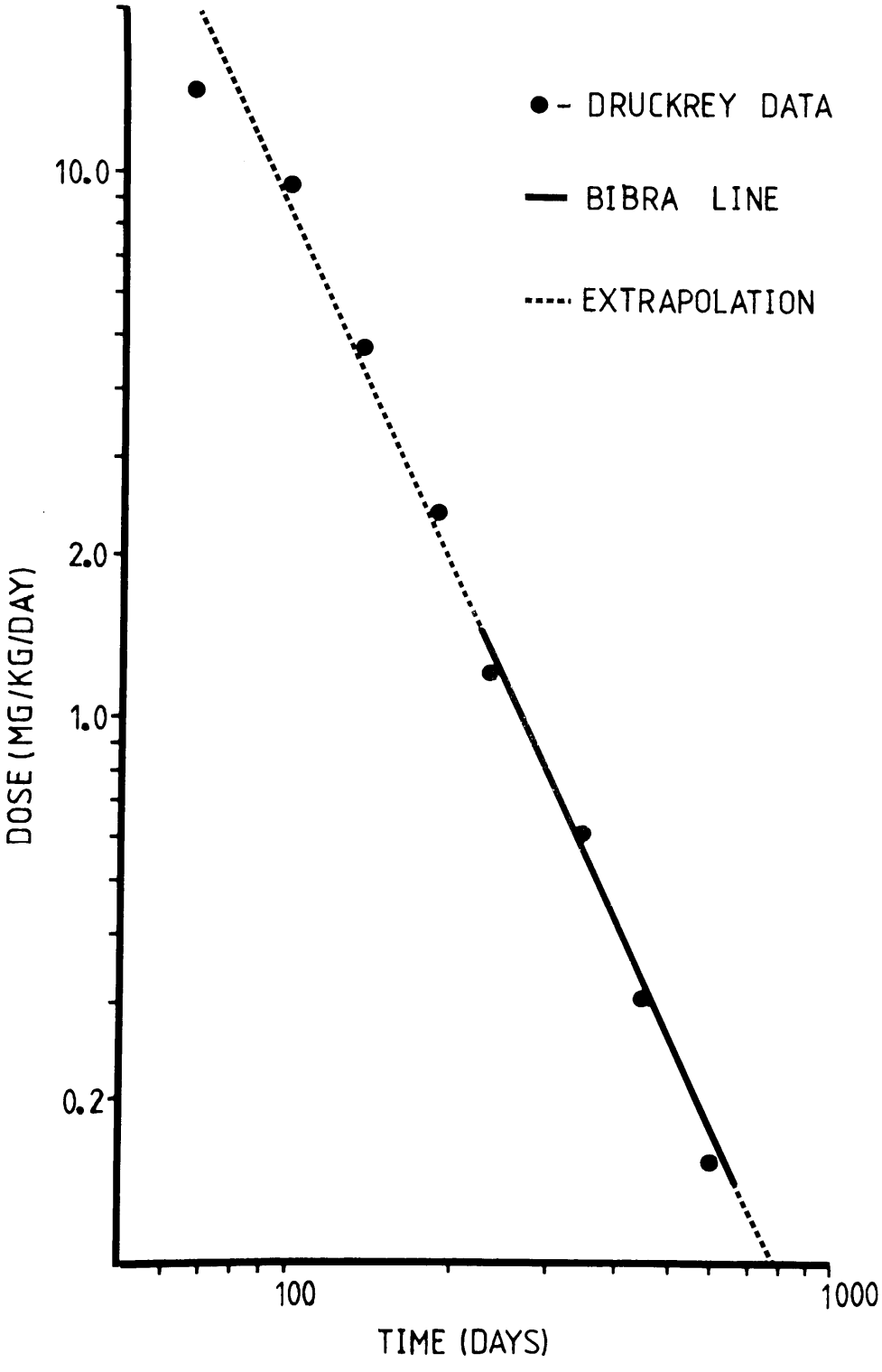
had tumours at both sites.

Tumour Time Related to Dose

The results of the studies with DEN are compared graphically in figure 6.1 with the data generated in an earlier study by Druckrey (1967). The median time of tumour data obtained by Druckrey are plotted on the same axes as the dose-response lines for DEN from this study. The closeness of fit would tend to suggest that rat strain plays little part in the pattern of dose-response. There is in addition little indication of any change in the relationship between dose and time of tumour over the whole range of doses studied.

The analysis of this study reported in chapter 5, indicates that the lack of a detectable effect at the lowest doses of this study could be explained by the inability, of groups of the size used, to detect the low incidences of tumours which might be expected. While this explanation is that which requires least assumptions it is by no means the only one that can be applied to these results. If it is assumed that the pattern of dose-response changes at a point just below the level of detectable effects then a wide range of conclusions can be drawn. Three alternative extrapolations are given in figure 6.2. Alternative (a) assumes that the relationship alters at low doses such that there is little effect of reducing dose on median time of tumour. Alternative (b) assumes that the experimentally determined relationship remains valid at all doses, while (c) assumes that the relationship alters at low doses to allow reducing dose to increase time of tumour at a greater rate than before.

FIGURE 6.1 A COMPARISON OF THE RESULTS OF DRUCKREY [1967] WITH THE DOSE-RESPONSE LINE FOR DEN IN FEMALE RATS FROM THE BIBRA STUDY



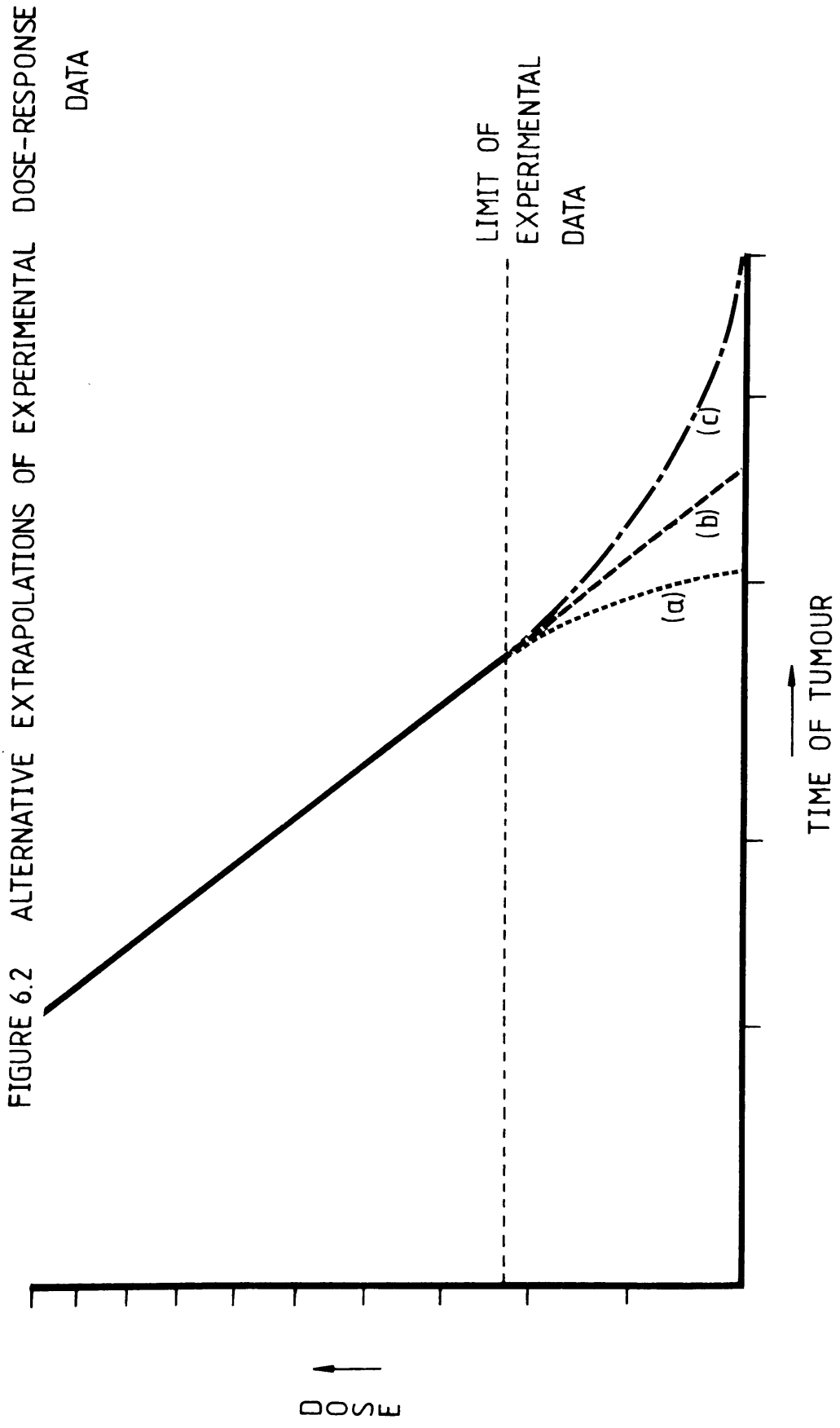


FIGURE 6.2 ALTERNATIVE EXTRAPOLATIONS OF EXPERIMENTAL DOSE-RESPONSE DATA

The only justification for choice in this situation is lack of information. There is no evidence that there is any change in the relationship at low doses thus alternative [b] has been assumed throughout analysis of this study, however, some consideration is given to other alternatives below.

Factors That May Modify Relationship

A wide range of influences may play a part in altering the pattern of dose-response at very low doses. The type of changes that may decrease the chance of carcinogenic change are:

[i] A metabolic shift from activation to detoxification at low doses [i.e. at high doses the detoxification pathway is saturated]

[ii] Ability of scavenger molecules such as glutathione to contain the quantity of radicals generated.

[iii] Ability of DNA repair mechanisms to keep up with the rate of damage.

There are however certain alternative changes that may act in the opposite direction:

[i] A metabolic shift towards greater proportional activation at low doses [i.e. that a first stage activation process has been saturated at high doses with the bulk of ingested nitrosamine being detoxified]

[ii] At high doses it is likely that DNA repair processes are induced by the carcinogen. At low doses the absence of such an induction may lead to a relatively greater effect from these doses.

Unfortunately, although there is evidence for several pathways in nitrosamine metabolism and effects on DNA repair no firm conclusions can be reached as to the direction of such effects or about the '*in vivo*' doses at which they may occur. It is therefore assumed that these factors act to cancel out each other's effects.

Alternative Approaches to Analysis of this Data

A wide range of models exist at present, all seeking to provide the best method of analysing carcinogenicity data (Peto, 1980; Hartley and Sielkin, 1977; Albert and Altschuler, 1973). Each of these methods has its basis in certain assumptions concerning the nature of chemical carcinogenesis and thus the mathematics of the relationship between dose and response. Over the observable range of tumour incidence all the models are likely to be capable of providing a good fit to the data. At the low doses, where any calculation of effect is based on extrapolation of the model, there is little to choose between the various approaches although each offer different conclusions. This study provided a unique opportunity to avoid the use of established models by an analysis guided by the biology of the process involved, where assumptions have been kept to the minimum necessary to provide an adequate summary or explanation of the data. An independent appraisal of this data is soon to be completed by Richard Peto of Oxford.

Extrapolation from Rat to Man

If the extrapolation from high to low dose in rats is considered to be uncertain the process of extrapolating

quantitatively from rat to man is almost totally a matter of guided guesswork.

Some thought needs to be given to the equivalence of exposure levels. In vitro metabolic studies by Montesano and Magee (1970) using DMN indicated that for this nitrosamine, under the conditions chosen, the rate of metabolism was similar for rat and man. As liver weight represents a roughly similar proportion of total body weight in the two species a dose administered as mg/kg body weight is likely to present a similar dose to each cell of the target tissue.

Generally the rate of xenobiotic oxygenation is linearly related to body-weight (Parke, 1982), and as such takes place more rapidly in rat than in man. As nitrosamines are activated by oxidative metabolism it is likely that activation of a given daily dose will be greater in rats than man, leading to earlier effects in the former species. It is possible however that the difference in lifespans of the two species may to a great extent compensate for these differences in metabolism when lifetime carcinogenic risks are considered. As it is not yet certain which enzyme is involved in nitrosamine activation it is difficult to put any quantitative relationship on the species differences in rates and extents of metabolic activation. Thus the same units of dose-rate can be used for rat and man.

A second consideration is the time taken for tumours to appear. It is likely, from other experience of the carcinogenic process, that the time from first treatment to first tumour at a given dose is not an absolute quantity

regardless of species. It is more likely that this time-period is in some way related to average life-span. It is proposed therefore to assume that median lifespan in the rat is equivalent to median human lifespan for the purposes of extrapolation.

Although a whole range of other factors may be involved in determining the response to nitrosamines it is considered that, in general, human diversity may well be as great as the species differences, thus little account of these factors can be taken in extrapolating from rat to man. It is considered that where species differences occur the rat is likely to represent the most adverse response that might be expected in a human population and is a valid model for calculating maximum expected human risks.

Calculation of Expected Human Risk

By its nature, the process of extrapolation from high-dose exposure in animals to low-dose human exposure is open to a variety of techniques each with their own merits. The extrapolation that follows is an extension of the analysis described previously and is based on a number of assumptions that are identified when appropriate.

The first stage of analysis is based on the assumption that the relationship established between dose and time of tumour for experimental doses, holds true for all doses. The regression lines obtained for the plots of median time of tumour against dose have been used to calculate median tumour times appropriate to doses of 0.143 and 1.43 μg/kg/day, these being equivalent to human exposure of 10 or 100 μg per

day at a body weight of 70 kg. (Table 6.1)

As might have been expected the predicted median times of tumour lie well beyond the normal lifespan of the animals used. However, the tumours at any dose are distributed in time. The pattern of this distribution was summarised from the experimental data in the Standard Curves (figures 5.7 and 5.8). Assuming that the pattern of the distribution continues to be appropriate at the doses equivalent to human exposure then the predicted cumulative incidence curves for these doses will be based on the following points (Table 6.2).

Due to the size of the experimental groups the derived curve for distribution around the median cannot directly be used to predict the time at which incidences lower than 2% are reached. If it is assumed that the risk approaches 0 at zero dose then the lower end of the distribution can be extrapolated to give some estimate of tumour risk within the normal lifespan. The regression of $\log_e$ Cumulative incidence on $\log_e$ time showed a correlation coefficient of > 0.99 in all cases and this relationship has been used to extrapolate the lower end of the cumulative distribution so that estimates of risk can be obtained for any age. The cumulative incidence of tumours expected at median lifespan and at the time that the last animal died is given in Table 6.3.

The predicted cumulative incidences within rat lifetimes can be used to estimate the magnitude of the human risk from these doses of nitrosamine. This use does however depend on the following assumptions:

Table 6.1 Estimates of median time of tumour by extrapolation from experimental data in rats

	Estimated median time of tumour† at a dose of	
	0.143 µg/kg/day	1.43 µg/kg/day
Male DEN	17323	6007
Female DEN	14980	5278
Male DMN	141337	27605
Female DMN	38017	10502

† - time in days from first dose of nitrosamine.

Table 6.2 Predicted distribution of tumours at very low doses of nitrosamines

Cumulative Incidence (%)	Time (days from first dose) that incidence reached			
	♂ DEN	♀ DEN	♂ DMN	♀ DMN
0.143 µg/kg per day				
2	12074	10441	80562	21670
10	14136	12224	105720	28437
20	15435	13347	120136	32314
30	16197	14006	128617	34595
40	16769	14501	134411	36154
50	17323	14980	141337	38017
1.43 µg/kg per day				
2	4187	3679	15735	5986
10	4902	4307	20649	7855
20	5352	4703	23464	8927
30	5617	4935	25121	9557
40	5815	5109	26252	9987
50	6007	5278	27605	10502

Table 6.3 Predicted cumulative incidence of tumours within rat lifespan at very low doses of DEN and DMN

Dose (μg/kg/day)	Cumulative incidence (%) at day	
	850	1300
0.143 DEN ♀	3.93×10^{-10}	1.75×10^{-8}
1.43 DEN ♀	4.40×10^{-6}	1.96×10^{-4}
0.143 DMN ♀	1.59×10^{-8}	1.84×10^{-7}
1.43 DMN ♀	2.64×10^{-5}	3.05×10^{-4}
Dose (μg/kg/day)	**Cumulative incidence (%) at day**	
	920	1300
0.143 DEN ♂	2.18×10^{-10}	4.79×10^{-9}
1.43 DEN ♂	2.81×10^{-6}	6.19×10^{-5}
0.143 DMN ♂	1.29×10^{-11}	9.49×10^{-11}
1.43 DMN ♂	1.58×10^{-7}	1.16×10^{-6}

(i) That the time taken for a given dose of nitrosamine to induce tumours is related to the natural lifespan of the species involved. For this purpose the median lifespan of rats and humans is assumed to be:

Sex	Rat	Human
Male	920 days	70 years
Female	850 days	75 years.

(ii) That there is no difference in sensitivity to the nitrosamines between man and rat, and that all the characteristics of the relationship between dose and time of tumour established for rats hold true for humans.

The cumulative incidence data cannot themselves be used to estimate human risk. First of all the expected incidence of tumours is calculated for the two intervals, 0 to median lifespan and median to end of lifespan. This is calculated as:

$$\text{Incidence of tumours expected within interval as (\%) of average numbers of animals alive in interval} = (I_E - I_S) \times \frac{100}{S_S - (\frac{S_S - S_E}{2})}$$

I_S = Cumulative Incidence at start of interval

I_E = Cumulative Incidence at end of interval

S_S = Survival at start of interval

S_E = Survival at end of interval.

The age distribution of the human population of the U.K. (CSO, 1980) shows the numbers of individuals alive in the

intervals corresponding to those used in Table 6.4 as:

0-Median	51.559×10^{6}	Median-End	4.346×10^{6}

From table 6.4 the extrapolated data for DMN-treated females indicates the greatest incidence of tumours, with a figure of 4.69×10^{-3} tumours/10^{6} individuals/year up to median lifespan and 0.444 tumours/10^{6} individuals/year over the remaining years. With the population age-distribution defined above this would indicate a total incidence of 2 tumours/year, in the whole U.K. population, due to nitrosamines.

As all the other extrapolations yield predicted incidences considerably lower than this it may be concluded, subject to all the assumptions identified, that exposure of the U.K. population to 100 µg/person/day of nitrosamines equivalent to DMN is unlikely to increase human death-rates by a detectable amount.

Conclusions

The magnitude of the calculated human risk may, if all the assumptions are agreed, provide some measure of assurance that current levels of exposure to nitrosamines are not a major cause of death in the U.K. population.

It is clear, however, that the risk is finite, which poses two questions. Firstly nitrosamines cannot be considered in isolation; it is known that two or more carcinogens having the same target organ can have an additive effect (Newberne and Connor, 1980). The predicted level of response to 100 µg/day of nitrosamine would leave the population on the threshold of response to a whole range of other carcinogens.

Table 6.4 Predicted incidence of tumours

Dose (µg/kg/day)	Predicted incidence (%) within interval	
	0 - MEDIAN	MEDIAN - END
0.143 DEN ♀	5.24×10^{-10} †	6.84×10^{-8}
1.43 DEN ♀	5.87×10^{-6}	7.66×10^{-4}
0.143 DMN ♀	2.12×10^{-8}	6.72×10^{-7}
1.43 DMN ♀	3.52×10^{-5}	1.11×10^{-3}
0.143 DEN ♂	2.91×10^{-10}	1.83×10^{-8}
1.43 DEN ♂	3.75×10^{-6}	2.36×10^{-4}
0.143 DMN ♂	1.72×10^{-11}	3.28×10^{-10}
1.43 DMN ♂	2.11×10^{-7}	4.01×10^{-6}

† Figures are the percentage of the average number of animals alive in the interval that can be expected to die from a treatment-related tumour in that interval.

A second thought, arising from the results of extrapolation relates to the end-point used in the animal studies described above. The time of tumour was the time at which the tumour brought about the death of the animal. No measure has been obtained, in this or any other study of the relationship between dose of carcinogen and tumour development time. The loss of a human life through cancer has its obvious sadness but it is the suffering that precedes death that has made cancer the dread of so many. No estimate is possible from this study of the amount or extent of suffering that may derive from nitrosamine exposure.

This study adds a step to the understanding of the risks posed by low levels of chemical carcinogens. Any further progress must come from a general understanding of the process of carcinogenesis and a detailed study of the fate '<u>in vivo</u>' of very low doses of carcinogens.

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